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Adaptive cellular mechanisms in response to Glutamine-starvation

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1. ABSTRACT

Glutamine (Gln) utilising cells suffer from Gln-starvation during critical illness when plasma Gln levels are decreased. This study investigates whether such cells activate adaptive mechanisms. Monocytic U937 cells were cultured at 0.6 and 0.2 mM Gln for up to four days. Within the first day a decrease of ATP (78% of control), intracellular free Gln (13%), Hsp70 (74%) and proliferation rate (79%) was observed. A prolonged culture at 0.6 mM Gln for additional three days led to a recovery of ATP (97%), Hsp70 (91%) and proliferation (92%). The intracellular free Gln increased only to 41%. At 0.2 mM Gln, however, all levels remained decreased. The activation of the metabolic sensor AMP activated kinase (AMPK) increased immediately in Gln-starving cells but regained normal values only in cells cultured at 0.6 mM. A proteomic analysis identified 23 proteins, which were affected by Gln starvation including metabolic enzymes, proteins involved in synthesis and degradation of RNA and proteins, and stress proteins. These data show that Gln-utilising cells activate adaptive mechanisms in response to Gln-starvation, which enable them to overcome a Gln shortage. At very low Gln concentrations, these adaptive mechanisms are not sufficient to countervail the lack of the amino acid.

2. INTRODUCTION

With a concentration of 0.5-0.6 mM, glutamine (Gln) is the most abundant free amino acid in human plasma. Due to this high concentration, Gln is a ready source of energy and is used besides glucose as a supplementary energy substrate for the intestine and in particular for monocytes/macrophages. 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 below 0.4 mM and monocytes suffer from Gln-starvation. Many organisms and tissues react to a reduced amino acid supply with activation of adaptive cellular mechanisms in order to overcome the shortage. The identified levels of regulation include transcriptional control, as well as mRNA stability and translational control. Numerous *in vitro* studies revealed that Gln-starvation induces many changes in phenotype and function of Gln-utilising cells. Gln depletion of primary monocytes and monocytic U937 cells for only 3 hours resulted in a reduction in proteasome-mediated proteolysis (1). This was accompanied by an accumulation of ubiquitin-protein conjugates and a reduction of intracellular ATP. Furthermore, U937 cells were more susceptible to the apoptotic triggers Fas-ligand and TNF-alpha 4 h after withdrawal of Gln (2). Studies in leukaemia-

derived CEM and HL-60 cells revealed that Gln-deprivation induces ligand-independent CD95 receptor signalling and apoptosis (3). In a recent publication, we could show that Gln-starvation of monocytic U937 cells for 4 h induces specific alterations in their proteome with the stress protein Hsp70 showing the strongest reduction (4). This decrease in Hsp70 expression was due to an enhanced decay of Hsp70 mRNA. All these studies clearly show that Gln-starvation induces a specific response immediately after withdrawal of the amino acid in primary monocytes as well as in monocytic U937 cells. However, it cannot be concluded whether these cells activate adaptive mechanisms in response to Gln, whether the described changes belong to such a mechanism and whether these cells are able to compensate for the lack of the amino acid. Some hints to an adaptive capacity of monocytic U937 cells come from earlier studies in which cells were exposed to Gln-starvation for up to five days (5). Although this treatment led to a reduced proliferation and an increased cell volume, the cells showed no impairment of their viability. Similarly, primary monocytes can be cultured in the absence of Gln for up to five days without any effect on their survival (6). Thus, these cells must somehow overcome the lack of Gln. The present study aims to investigate whether Gln-utilising cells are able to adapt to Gln-starvation and to characterise such a response. Therefore, we analysed monocytic U937 cells exposed to different degrees of Gln-starvation for up to four days. This long-term Gln-starvation approach allowed the analysis of the immediate cell response as well as of the succeeding adaptation to the reduced Gln supply. We could clearly show that U937 cells are able to compensate for mild Gln-starvation and could identify proteins involved in this process by a proteomic analysis.

3. MATERIAL AND METHODS

3.1. Chemicals

Radiochemicals were from Amersham Bioscience (Uppsala, Sweden), o-phtalaldehyde, potassium borate solution (pH = 10.4), and ß-mercaptoethanol were purchased from Pierce (Rockford, IL, USA). All other chemicals used were obtained from Sigma (St. Louis, MO, USA) if not specified. All chemical were p.a. grade.

3.2. 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 % 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 with reduced Gln-supplementation was used. Without any supplemented Gln the medium still contained 0.05 mM Gln deriving from FCS.

3.3. Analysis of intracellular glutamine

Cells were washed in PBS, counted, $5x10^6$ cells were suspended in 100 µl 3 % 5-sulfosalicylic acid containing 100 µM ß2-thienylalanine and the suspension was shock-frozen and defrosted. After centrifugation, the amino acids in the supernatant were analysed by HPLC: 1 volume of OPA-reagent (13 mg o-phtalaldehyde, 200 µl methanol, 800 µl 1 M potassium borate pH = 10.4, 25 µl ß-mercaptoethanol and 9 ml water) was added and 60 seconds later the sample was loaded onto a 150 x 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, Tokyo, Japan). Separation was 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.s 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.). Fluorescence output (λ_{ex} = 330 nm; λ_{em} = 408 nm) was quantified by comparison to the internal standard (ß2-thienylalanine) as well as to data obtained for a standard mixture of amino acids.

3.4. Western blot analysis

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, 1 µg/ml leupeptin, and 1 µg/ml aprotinin) combined with short sonification at 0°C. After centrifugation (10 min, 15,000 *g*, 4°C) the supernatant was stored in aliquots at -70°C. The cellular protein content was measured using a Bradford assay (Pierce, Rockford, IL, USA). For Western blot analysis, 10 µg samples were separated by SDS-PAGE transferred to a nitrocellulose membrane by electroblotting. Hsp70 expression was revealed with an Hsp70 specific antibody (SPA-810, Stressgen, Victoria, Canada). The levels of AMPK and phospho AMPK were determined by specific antibodies (Upstate, Charlottesville, VA, USA).

3.5. Analysis of intracellular ATP

The intracellular ATP level was determined using the bioluminescence luciferin/luciferase assay system with modifications as previously described (7). Briefly, U937 cells were washed twice in PBS, counted and 1 ml 0.5 M $HClO_4$ was added to 10^6 cells. After centrifugation (14,000 g for 10 min) 50 µl of supernatant was mixed with 12.5 µl 1 M Na_2CO_3 to elevate the pH. Then 937.5 µl TAE (100mMTris–acetate pH = 7.8, 2 mM EDTA) were added. Two hundred microliters of this solution was filled in a transparent vial and after automatic injection of 50 µl ATP monitoring reagent (Bio-Orbit, Turku, Finland), light emission was measured for 20 s in a chemiluminometer (Auto-Lumat LB953, Berthold, Wildbad, Germany).

3.6. Analysis of intracellular glutathione

Cells were harvested and washed twice with ice cold PBS. Two x 10^6 cells were mixed vigorously with 1 ml of an aqueous 6.5 % sulfosalicylic acid (Merck, Darmstadt, Germany). After 15 min incubation on ice, the supernatant was snap frozen in liquid nitrogen and stored at -70°C until further analysis by HPLC. Hundred microliters of the neutralised sample or glutathione (GSH) standard

were mixed with 100 µl MBB (0.57 % in acetonitril and sodium *N*-ethylmorpholine, pH 10.1), and allowed to react in the dark for 5 min before the reaction was stopped by the addition of 10 µl 50 % sulfosalicylic acid (8). The HPLC separation of low molecular mass thiol-bimane adducts was achieved on a Supelcosil LC-18 octadecyl-silyl silica column (150 mm X 4.6 mm, 3 µm particle size; Supelco, Bellefonte, PA, USA) , followed by fluorimetric detection (EP920 fluorescence detector; Jasco, Osaka, Japan) at an exication wavelength of 394 nm and an emission wavelength of 480 nm. Elution solvent A was composed of aqueous 9 % acetonitril, 0.25 % acetic acid and 0.25 % perchloric acid. The pH was adjusted to 3.7 with 40 % sodium hydroxide. Elution solvent B was based on a 75 % aqueous solution of acetonitril. The gradient elution consisted of 100 % A for 7 min., followed by 100 % B to elute matrix interferences, and returning to solvent A for re-equilibration for 12 min. at a flow rate of 1.0 ml/min. The resultant profiles were quantified on a basis of peak areas and compared with external standards of GSH (usually 0.625-10 µmol/l).

3.7. Cell lysis and determination of protein concentration

Proteins were extracted and quantified as previously described (9). Briefly, 1500 µl of cell suspension were mixed with 500 µl of ice-cold 6.1 N TCA solution containing 80 mM DTT (Roche, Mannheim, Germany). The mixture was incubated for one hour at 4°C to allow protein precipitation to complete. Then the extract was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was discarded and the pellet washed four times with 1,500 µl of ice-cold acetone each, containing 20 mM DTT; the pellet was regained in each step by centrifugation at 10.000 g. Thereafter the centrifuged pellet was dried by air evacuation. The pellet was resolubilized in denaturing 2D sample buffer containing 7M urea, 2M thiourea, 4 % CHAPS, 30 mM Tris-HCl (pH=8.5) by shaking overnight at 4°C. The protein concentration in resolubilized samples was determined in triplicates using a Coomassie protein assay kit with BSA as the standard protein (Pierce, Rockford, IL, USA). With appropriate pre-dilution, 2D sample buffer components do not interfere with the protein assay. Therefore, samples were diluted 1:20 with PBS and 5% of 2D sample buffer were added in the BSA standards.

3.8. Analysis of cell proteins by one and two dimensional gel electrophoresis

For two-dimensional gel electrophoresis (2DE) proteins were separated in the first dimension by isoelectric focusing (IEF) and in the second dimension by SDS-polyacryl amide gel electrophoresis. IEF was performed loading 120 µg of proteins by in-gel rehydration in a volume of 450 µl denaturating 2D buffer (7 M urea, 2 M thiourea, 4% CHAPS, 70 mM DTT, 0.5 % servalyt™ pH 3-10; Serva, Heidelberg, Germany) onto 24 cm IPG DryStrip pH 3-7 linear (Amersham Bioscience, Uppsala, Sweden) and focused for 50 kVh using an Amersham IPGphor unit. Before loading onto SDS polyacrylamide gels, IPG strips were incubated for 15 min in equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS) containing 1 % DTT and then for another 15 min in equilibration buffer containing 2.5% iodoacetamide. SDS polyacrylamid gels (260 x 200 x 1 mm, T = 11 %, C = 2.6 %) were cast according to Laemmli (10). The protein spots were visualised by a ruthenium II tris (bathophenantroline disulfonate) staining (11). Comparative spot pattern analysis was accomplished with Delta-2D software (Decodon, Greifswald, Germany). One-dimensional SDS-gel electrophoresis was performed with 130 x 160 x 1.5 mm gels.

3.9. Protein identification

The spots of interest were excised manually using a stainless steel scalpel and subjected to in-gel digestion procedure (12) using trypsin (from bovine pancreas, modified; sequencing grade, Roche, Mannheim, Germany). The solutions of tryptic peptides, extracted out of the gel pieces, were desalted and purified utilising ZipTip® technology (C_{18} reversed phase, standard bed Millipore, Bedford, MA USA) (13). The sample preparation for MALDI mass spectrometry was carried out on a stainless steel target, applying thin layer preparation technique (14) using α-Cyano-4-hydroxy-cinnamic acid (Prod-nr. C2020, Sigma Aldrich, St. Louis, MO, USA) as matrix dissolved in acetone (6 mg/ml). Positive ion mass spectra were recorded on a prototype vacuum MALDI TOF/curved field reflector TOF instrument (Shimadzu Biotech-Kratos Analytical, Manchester, UK) equipped with a nitrogen-Laser (λ=337 nm) by accumulating up to 500 single unselected laser shots. The instrument was operated in the reflectron mode, applying 20 kV acceleration voltage and delayed extraction (optimal setting for m/z 2000). External calibration was performed using an aqueous solution of standard peptides (Bradykinin fragment 1-7, human Angiotensin II, ACTH fragment 18-39). The lists of m/z-values derived from the mass spectra of in-gel digestions were submitted to the publicly accessible peptide mass fingerprint (PMF) search engines MS-Fit (15) and MASCOT (16) to search the SWISSPROT protein sequence database (Vers. 48.1 – 48.3) (17) and the non redundant database from NCBI (Release 2005/06/01) (18) applying restrictions for species (*homo sapiens*), molecular weight (value estimated from 2D gel ± 33%), isoelectric point (value estimated from 2D gel ± 2) and mass tolerance (± 0.35 Da). A PMF search result was considered only to be a correct identification if the protein was rated as a significant match in the context of the reliability scoring system of both search algorithms. To confirm each identification result, post source decay (PSD) or low energy collision induced dissociation (CID) MS/MS experiments were performed on selected peptides. PSD experiments were performed on the high vacuum MALDI TOF/curved field reflector TOF instrument using the same sample preparation procedure as described previously. Low energy CID experiments were performed on a HCT^{plus} 3D-ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a second generation atmospheric pressure (AP)-MALDI pulsed dynamic focusing (PDF)-source (Agilent Technologies, Palo Alto, CA, USA) and a nitrogen laser (λ=337 nm). The eluate from ZipTip® purification was prepared onto a gold coated target using the dried droplet technique (19) The eluate from ZipTip® purification was prepared onto a gold coated target using the dried

droplet technique (6 mg/ml). Positive ion AP-MALDI mass spectra were obtained using the following parameters: 350°C dry gas temperature and 100 ms accumulation time. Precursor ions for low energy CID-experiments were selected with an isolation width of 4 Da. External calibration was performed using the same standard peptide mixture as described above. The minimum requirement for successful confirmations of the PMF result was either at least one tryptic peptide yielding enough fragment ions to facilitate the manual assignment of a consecutive amino acid sequence, or two tryptic peptides, which did not allow the readout of a sequence tag, but reached significant MOWSE scores when submitting the list of m/z values to MASCOT MS/MS ion search (16). These searches have been performed using restrictions for species (*Homo sapiens*), database (SwissProt, NCBInr), precursor and product ion tolerance (PSD-data: ± 1 Da, low energy CID-data: ± 0.5 Da).

4. RESULTS

4.1. Effect of long-term Gln-starvation on cellular metabolism

To evaluate the effect of long term Gln-starvation on cellular metabolism cells were cultured in a medium supplemented with different Gln concentrations (2.0 mM, 0.6 mM, 0.2 mM, and 0.0 mM Gln) for up to 4 days. Since U937 cells show optimal growth at 2 mM Gln, this concentration was used as control. The intracellular level of free Gln (Gln_i) was measured before start of the experiment and at every single day. As shown in Figure 1A, at 2.0 mM Gln in the medium the intracellular Gln concentration was slightly below 6000 nmol Gln per µg protein. This level remained stable during the four days. In contrast, at reduced extracellular Gln the intracellular level dropped down immediately below 1000 nmol Gln per µg protein within 24 h. Only cells cultured in presence of 0.6 mM Gln were able to increase their Gln_i after 48 h to about 35% of control. However, there was no further increase observed in the following days in these cells. The Gln_i of cells cultured at 0.2 mM or 0.0 mM Gln remained below 5% of control for the whole experiment. These data clearly showed that extracellular Gln influenced strongly the intracellular concentration of free Gln. Only at 0.6 mM Gln, cells seemed to activate successful compensative mechanisms to increase their Gln_i. The next experiment investigated the effect of Gln-starvation on cell proliferation. Cells were cultured in medium with 2.0 mM, 0.6 mM, 0.2 mM, and 0.0 mM Gln for up to 96h. Cells were collected every 24 h, counted and seeded freshly with a density of 5×10^5 cells per ml. This helped to avoid any influence of cell density. The daily increase in cell number (daily replication rate) was used as a measure for cell proliferation with 1 indicating no proliferation. The results are shown in Figure 1B. At 2.0 mM Gln, the daily cell replication rate remained stable at a level slightly above 2.2. This indicated that cell number increased by this factor within 24h. At reduced Gln concentration, daily replication rate declined to about 1.6 within 24h. Cells cultured at 0.6 mM Gln returned to a normal daily replication rate within the following 24h. At lower Gln levels, the daily replication rate remained reduced for the rest of the experiment. It has to be mentioned that the daily replication rate was never below 1.4, even at 0.0 mM Gln. This indicated that also in the absence of Gln a cell proliferation occurred. Next, we investigated the effect of Gln-starvation on the intracellular concentration of ATP. Cells were cultured in medium with 2.0 mM, 0.6 mM, 0.2 mM, and 0.0 mM Gln for up to 96h and ATP was measured every day. As shown in Figure 1C, the availability of Gln had a strong influence on ATP level. At 2.0 mM Gln the ATP level remained unchanged during over 96h. At 0.6 mM Gln, ATP levels declined to 80% of normal within the first day and remained reduced until 72h. Then the ATP level increased to normal in the last day. At 0.2 mM Gln, ATP declined in the first day to 58% of normal and remained always below 68% throughout the experiment. At 0.0 mM Gln, ATP levels declined in the first day down to 44% of normal. In the following days, it decreased further with the lowest level at 32% of normal after 3 days. Taken together, these experiments show that the effect of Gln-starvation on cellular metabolism depends on the level of still available Gln. Cells seem to activate mechanisms for compensation of the missing amino acid. However, only cells exposed to mild Gln-starvation (0.6 mM Gln) are able to overcome the lack of Gln to a certain extent.

4.2. Effect of long-term Gln-starvation on cell protective mechanisms

Decreased intracellular ATP levels result in an increase of AMP, which is known to activate the AMP-activated kinase (AMPK) in a variety of cells by phosphorylation. Activated AMPK finally down-regulates energy consuming processes and stimulates ATP production to protect cells from starvation. To investigate whether Gln-starvation is able to activate AMPK we exposed cells to 2.0 mM, 0.6 mM, and 0.2 mM Gln for up to 96 h. Cells were harvested after 24 h and after 96 h and AMPK-phosphorylation was investigated by Western blotting. As Shown in Figure 2, Gln-starvation resulted in an increased phosphorylation of AMPK within 24 h. The level of phosphorylation depended on the degree of Gln-starvation. After 96 h the AMPK phosphorylation returned to normal in cells exposed to 0.6 mM Gln. In contrast, AMPK phosphorylation remained high in cells exposed to 0.2 mM Gln. In a recently published study, we could show that short-term Gln-starvation results in a specific reduction of stress protein Hsp70 expression by reduced mRNA stability (4). To evaluate the effect of long-term Gln-starvation on Hsp70 expression we cultured cells at 2.0 mM, 0.6 mM, and 0.2 mM Gln for 24 h and 96 h. Then Hsp70 expression was determined by Western blotting. As shown in Figure 3, Hsp70 expression declined at reduced Gln supply within 24 h to about 75% of normal. However, after 96 h Hsp70 expression was found to increase to normal levels in cells cultured at 0.6 mM Gln while it remained decreased at 0.2 mM.

4.3. Effect of long-term Gln-starvation on overall protein synthesis

Gln is, as an amino acid, a substrate for protein synthesis. Therefore, we investigated the effect of long-term Gln-starvation on protein synthesis rate and cellular protein content. Cells were cultured in medium with 2.0 mM, 0.6 mM, 0.2 mM, and 0.0 mM Gln for 24 h and

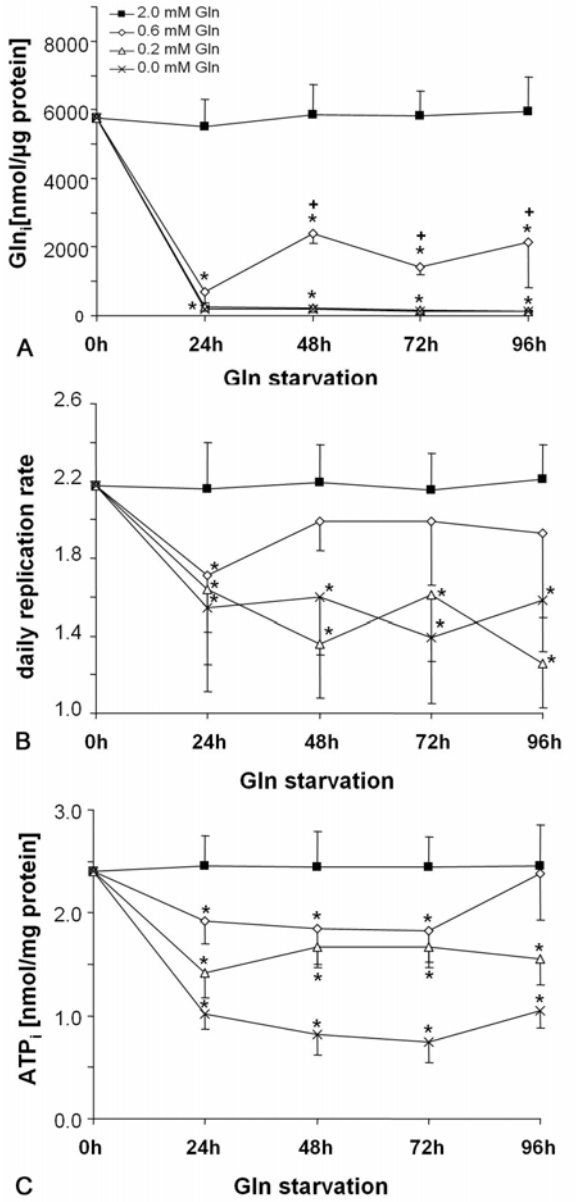

Figure 1. Influence of extracellular glutamine supplementation on cellular metabolism. U937 cells were cultured in medium supplemented with various concentrations of Gln for 24-96h. Cells were freshly seeded each day and density never exceeded $0.5x10^6$ cells/ml. Apoptosis or necrosis were excluded by trypan blue stain (viability was always ≥ 95.6%). At each time point, cells were harvested and analysed. (A) Intracellular free Gln (Gln_i). Gln_i was measured by HPLC and set into relation to total cellular protein. Data are mean ± SD of n = 5. (B) Daily replication rate. Cells were counted every day and the duplication rates within the last 24 h were calculated. Data are mean ± SD of n = 8. (C) Intracellular ATP levels (ATP_i). ATP_i concentrations were determined by a luminescence assay and set into relation to total cellular protein. Data are mean ± SD of n = 5. Statistical analysis is an one-way ANOVA with contrast against control (2mM Gln; significance indicated with an asterisk; Dunnett, 95% CI did not include 0) or against 0.6mM Gln (significance indicated with a cross).

96 h. To measure the cellular protein content cells were lysed, free amino acids were removed by TCA precipitation and protein amount in the precipitate was measured. As shown in Figure 4A, Gln-starvation had no effect on protein content of cells. To investigate the influence of Gln-starvation on the overall protein synthesis rate, cells were cultured under the same conditions as above and 2 h before end of the experiment [^{35}S]-Met was added for metabolic protein labelling. Then cells were lysed and proteins were separated in a SDS-gel electrophoresis. The autoradiogram of the gels indicated the [^{35}S]-Met incorporation into the proteins. We took the intensities of the autoradiogram between 20 kDa and 150 kDa as a measure of overall protein synthesis. As shown in Figure 4B, there was no effect of Gln-starvation on overall protein synthesis rate.

4.4. Effect of long-term Gln-starvation on the proteome

To evaluate whether Gln-starvation influences the expression of individual protein species without affecting the overall protein synthesis rate we used a proteomics approach. Cells were cultured in medium with 2.0 mM, 0.6 mM, and 0.2 mM Gln for 24 h and 96 h. Then proteins were extracted and separated by two-dimensional gel electrophoresis. Correspondingly, the resulting gels were termed: 2.0/24, 0.6/24, 0.2/24, 2.0/96, 0.6/96, and 0.2/96. Protein spots in the gel were visualised by ruthenium II tris (bathophenantroline disulfonate) staining followed by digital fluorometric scanning. For quantification of spot intensities, the fluorescence signal intensities within the spot boundaries were used to calculate the fluorescence intensity volume (FIV) of each spot. An average of 595 spots (506-672) was detected in a typical gel (Figure 5). About ninety percent of these spots (532 spots) were found repeatedly in every 2.0 mM gel. Only these spots were used for further analysis. To minimise gel-to-gel variations, the FIV of every single spot in a gel was normalised by dividing it by the sum of all FIV values of all spots in the gel (FIV_{all}). The resulting percentage fluorescence intensity volume (%FIV) was used as a measure for protein spot abundance. The scatter blot in Figure 6 compares this abundance of all 535 protein spots in the 2.0/24 gel with their abundance in the 2.0/96 gel. The comparison nicely showed that all spots had nearly the identical abundance in both gels. This indicated that at 2.0 mM Gln the protein expression profile does not change during time. Therefore, the $\%FIV_{2.0/24}$ was used as normal control to reveal the effect of Gln-starvation on protein spot abundance. The %FIV values of gels from Gln-starved cells (0.6/24, 0.2/24, 0.6/96 or 0.2/96) were divided by the %FIV values of the corresponding spot in the control gel ($\%FIV_{2.0/24}$). The $\log_2$ value of these ratios visualises a stimulatory or inhibitory effect of Gln-starvation in comparison to control: a $\log_2$ value of +1 indicates an increase by factor 2 and a $\log_2$ value of -1 a decrease by factor 2, whereas a $\log_2$ value of zero indicates no change (Figure 7). The $\log_2$(ratios) are plotted against the spot abundance. Most protein spots group in the y-axis around zero. Their deviation from zero corresponds to the expected normal distribution. This clearly shows that they are unaffected by a Gln-starvation. In addition, the $\log_2$(ratio) of these spots is independent of the spot abundance (x-axis). This excludes any artificial influence of the 2D gel

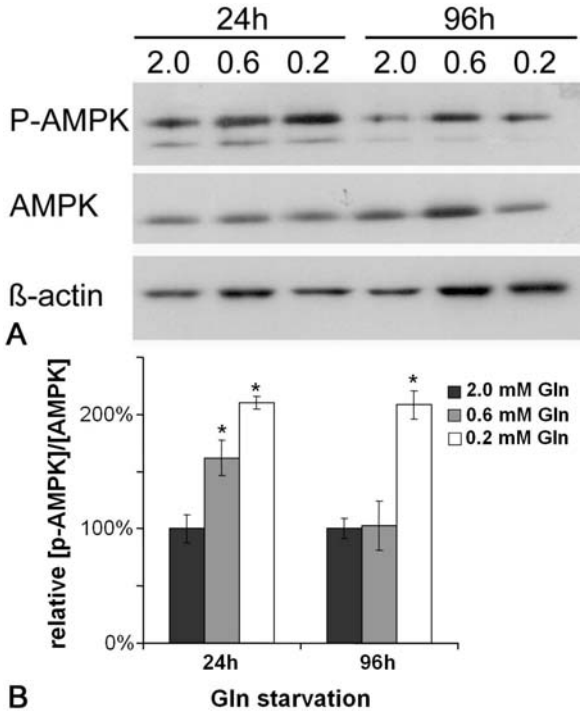

Figure 2. Influence of Gln-starvation on AMP-activated Protein Kinase (AMPK). U937 cells were cultured in medium supplemented with various concentrations of Gln for 24 and 96h. At both time point, cells were harvested and expression of AMPK as well as the level of phosphorylated AMPK (thr172, P-AMPK) was determined by Western blot. (A) Western blot analysis. The upper panel shows the expression of phosphorylated AMPK, the middle panel shows the level of whole AMPK, and the lower panel shows ß-actin levels for loading control. (B) Statistical evaluation. The Western blot signal of five independent experiments were quantified by densitometry and statistically evaluated. AMPK activation is expressed as mean ratio of P-AMPK in relation to total expression of AMPK. The mean ratio of control cells (2.0 mM Gln) was set to 100%. The asterisks indicate $p < 0.05$ compared to control cells as calculated by Students t-test.

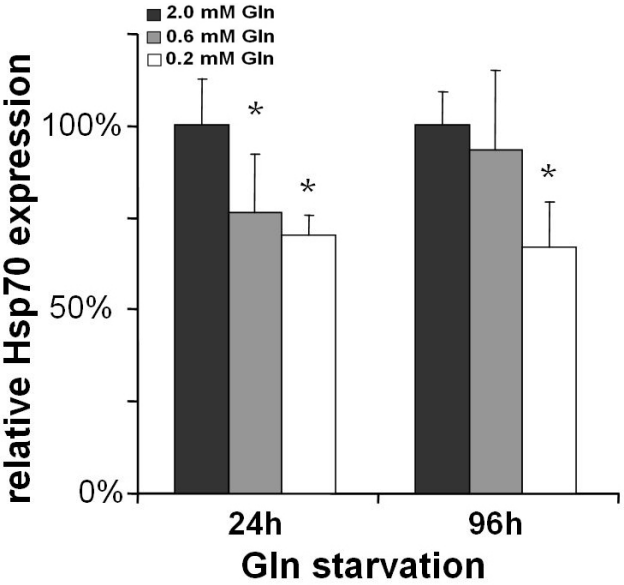

Figure 3. Influence of Gln-starvation on Hsp70 expression. U937 cells were cultured in medium supplemented with various concentrations of Gln for 24 and 96h. At both time points, were harvested and expression of Hsp70 was determined by Western blot. The mean Hsp70 in control cells (2.0 mM Gln) was set to 100%. The asterisks indicate $p < 0.05$ compared to control cells as calculated by Students t-test (n = 3).

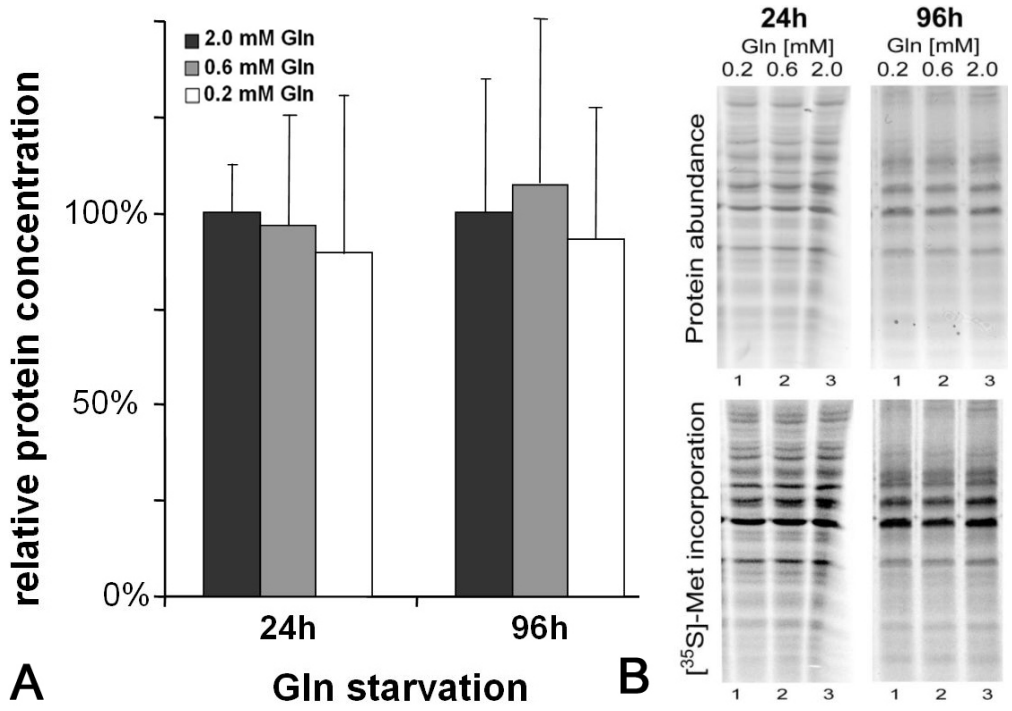

Figure 4. Cellular protein content and synthesis in Gln-depleted U937 cells. U937 cells were cultured in medium supplemented with various Gln concentrations for 24h or 96h. (A) Cellular protein content. At both time points, cells were harvested and free amino acids were removed by precipitation with 20% trichloroacetic acid at 0°C for 1h. After resolubilisation of the protein-pellet, protein content was determined by a Bradford assay. The mean protein content (n = 8) in control cells (2.0 mM Gln) was set to 100%. (B) Overall protein synthesis. Two hours before the end of the experiment [S^{35}]-Met was added to the cell suspension for incorporation into the synthesised proteins. At the end of the experiment, cells were harvested and a SDS polyacrylamide gel electrophoresis was performed. The abundance of the separated proteins was visualised by staining with ruthenium II tris (bathophenantroline disulfonate) (upper two panels). The [S^{35}]-Met incorporation, which is a measure for protein synthesis, was visualised by autoradiography (lower two panels).

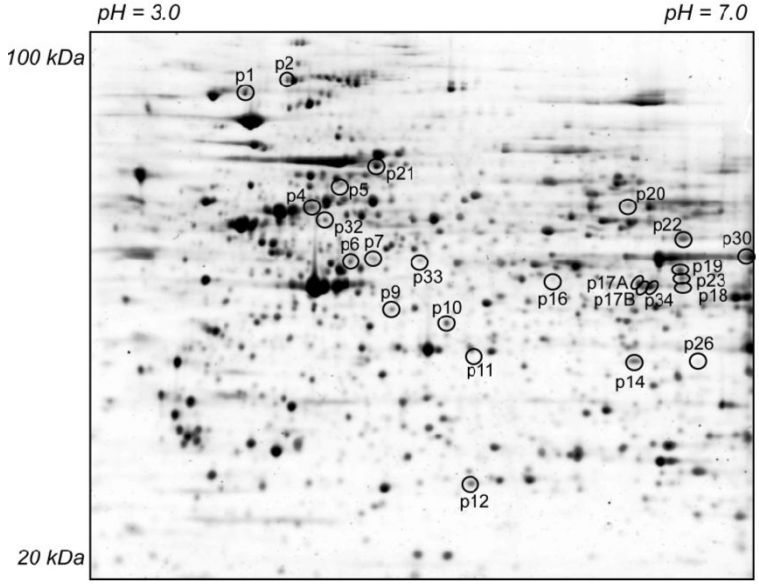

Figure 5. Two-dimensional gels of proteins from U937 cells. Cells were lysed and proteins were separated by two-dimensional gel electrophoresis. Gel were stained with ruthenium II tris (bathophenantroline disulfonate). First dimension: pH range from 3 to 7. Second dimension from 20 kDa to 100 kDa. The circles indicate the position of the protein spots listed in tables 1 and 2.

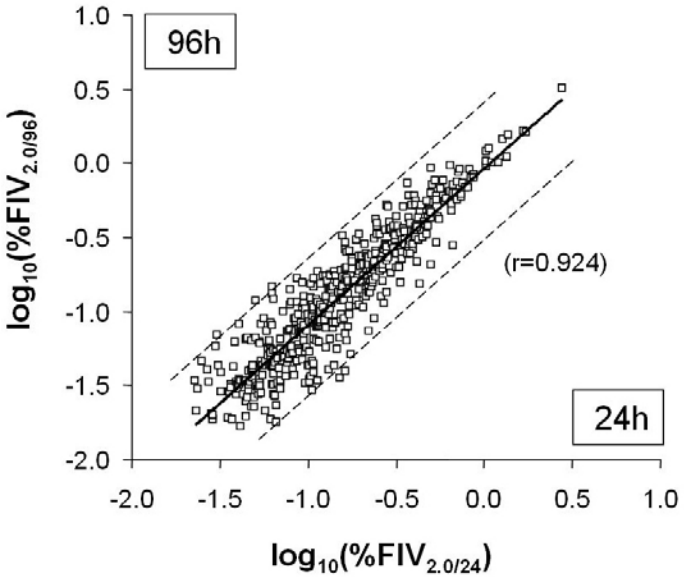

Figure 6. Scatter blot comparing the protein abundance in cells cultured for 24 h or 96 h at 2.0 mM Gln. Cells were harvested and proteins were separated by 2D gel electrophoresis. Protein spots were stained with ruthenium II tris (bathophenantroline disulfonate) and the fluorescence intensity volume (FIV) of every spot was measured. Every cross in the diagram indicates the spot abundance (expressed as $\log_{10}$ value of %FIV) in the 2.0/24 gel (X-axis) and in the 2.0/96 gel (Y-axis).The straight line represents the linear regression, the broken lines represents the 99% coincidence intervals. The correlation coefficient is indicated in brackets.

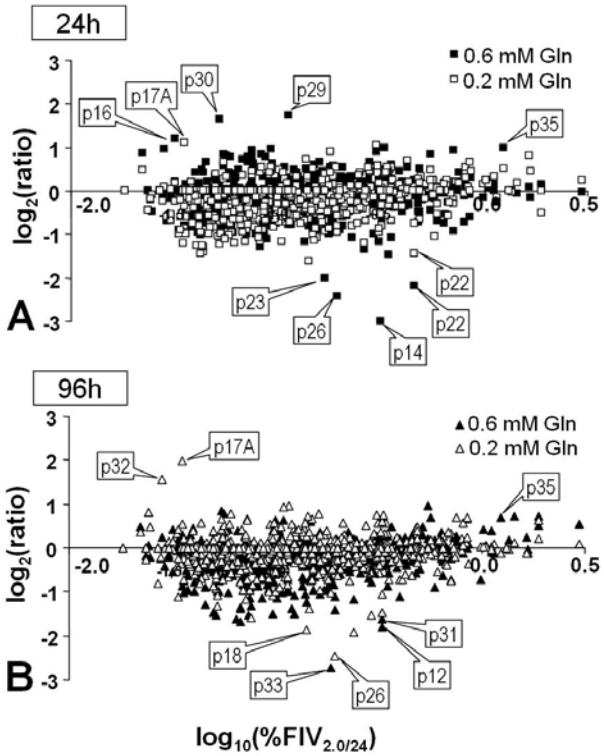

Figure 7. MA blots showing differences in the expression profiles of Gln depleted cells (0.6 mM Gln or 0.2 mM Gln) in comparison to cells cultured in complete medium (2.0 mM Gln). U937 cells were cultured in medium supplemented with 2.0 mM, 0.6 mM and 0.2 mM Gln for 24h (A) and for 96h (B). Then proteins were extracted and separated in a 2D gel electrophoresis. Protein spots were visualised by ruthenium II staining and the %FIV was used as a measure for abundance of every single spot. The MA blot shows on the Y-axis the $\log_2(\%FIV_{0.6 \text{ or } 0.2}/\%FIV_{2.0})$ and in the X-axis the $\log_{10}(FIV_{2.0/24})$ values. The labelled spots exhibit an disproportional high or low $\log_2$(ratio). Their position on the 2D gel is shown in Figure 6.

Table 1. Protein spots significantly increased or decreased by Gln-starvation

Spot ID	log2(Ratio to 2mM Gln)[1]			
	0.6mM Gln		0.2mM Gln	
	24h	96h	24h	96h
p1	**-1,16**	-0,08	-0,52	0,76
p2	-	-0,16	-0,64	-
p4	**-1,09**	-0,53	-0,8	-0,76
p14	-3	-0,32	-0,3	-0,15
P17A	-	0,29	**1,11**	1,99
P17B	-	0,18	0,17	-0,58
p18	-0,82	-1,24	**-1,62**	-1,87
p19	**-1,36**	-0,4	**-1,19**	-0,24
p20	-0,89	-0,66	**-1,08**	-0,27
p21	**-0,4**	0,21	**-0,43**	0,11
p22	**-2,19**	-0,31	**-1,43**	-0,63
p23	**-2,02**	-0,43	-0,93	-0,57
p16	**1,2**	-0,55	0,18	-0,04
p32	**0,96**	0,24	0,1	**1,55**
p34	**0,69**	-0,04	0,18	-0,16
p26	**-2,44**	-1,4	-0,82	**-2,45**
p30	**1,66**	-	-0,18	-0,24
p5	0,2	**-1,39**	-0,38	-
p6	-0,31	**-1,53**	**-1,06**	-0,92
p7	0,37	**-1,05**	0,28	-0,28
p11	0,44	**-0,46**	0,15	-0,1
p12	0,47	**-1,79**	0,08	**-1,04**
p33	-0,92	**-1,69**	-0,43	-0,42

[1] Values correspond to the logarithm on base 2 of the ratio between the abundance (%FIV) of protein spots from Gln starved cells (0.6 mM Gln or 0.2 mM Gln) with that of spots from control cells (2.0 mM Gln). The experiment was performed two times. The listed protein spots fulfil one of the following inclusion criteria: a $\log_2$(ratio) higher than +1 or lower than -1 in both experiments, or a log(ratio) higher than 0.4 or lower than -0.4 which was nearly identical in both experiments (max. error 10 %), or absence of the spot in the Gln-starvation group in both experiments. The values which fulfil these conditions are indicated in bold. Spots are sorted according to their expression levels as described in the discussion section.

Table 2. Protein identification results

Spot ID	SwissProt Acc. Nr.	Protein Name	Spot position		Theoretical[1]		Sequence Coverage[2]	MOWSEScore PMF[3]	Confirmation by PSD or CID MS/MS
			MW [kDa]	pI	MW [kDa]	pI			
			100	5.2	94	5.2			
p4	Q12874	Splicing factor 3A subunit 3 (SAP 61)	60	5.3	59	5.3	17 %	63	PSD
p5	-	n.i.							
p6	P52597	Heterogeneous nuclear ribonucleoprotein F (hnRNP F)	45	5.5	46	5.4	26 %	125	PSD
p7	-	n.i.							
p11	Q96C86	Scavenger mRNA decapping enzyme (DcpS)	35	6.0	39	5.8	16 %	83	CID
p12	-	n.i.							
p14	P40925	Malate dehydrogenase (MDH)	35	6.5	36	6.9	40 %	170	PSD
p16	-	n.i.							
p17A	P49411	Elongation factor Tu, (EF-Tu)	42	6.5	50	7.3	30 %	139	CID
p17B	P49411	Elongation factor Tu, (EF-Tu)	42	6.5	50	7.3	33 %	117	PSD
p18	-	n.i.							
p19	P52209	6-phosphogluconate dehydrogenase (PGDH)	45	6.8	53	6.9	20 %	113	PSD
p20	-	n.i.							
p21	P08133	Annexin 6 (A6)	70	5.5	76	5.4	26 %	155	PSD
p22	P00367 P25705	Glutamate dehydrogenase 1 (GDH) ATP Synthase α-chain (ATP-Sα)	55 55	6.6 6.6	61 60	7.7 9.2	23 % 19 %	90 86	PSD PSD
p23	P62195	26S protease regulatory subunit 8 (p45/SUG)	42	6.6	46	7.1	31 %	156	---
p26	-	n.i.							
p30	-	n.i.							
p32	-	n.i.							
p33	-	n.i.							
p34	P22234	Phospho ribosyl amino imidazole-succino carboxamide synthase (PUR6)	42	6.5	47	7.1	22 %	88	CID

[1]Calculated from sequences retrieved from SwissProt database using expasy "Compute pI/Mtool", http://www.expasy.org/tools/pi_tool.html,[2]Calculated as number of amino acids spanned by found peptides divided by sequence lengthof mature protein,[3]Probability based MOWSE Score for the PMF search result as calculated by MASCOT algorithm. Scores > 60 reflect significant results

analysis on the result. In contrast, some protein spots show disproportionately high or low ratios (e.g. in Figure 7A, 0.6 mM Gln: spots p16, p30, p29 and p35 are increased and spots p23, p26, p14, and p22 are decreased). This result showed that although the expression of most proteins was unaffected by Gln-starvation the expression of some proteins was specifically increased or decreased. The list of protein spots which expression was significantly influenced by Gln-starvation is shown in table 1. The quantitative evaluation indicated that different protein species reacted highly specific. About 5 protein spots were increased within 24h at 0.6 mM Gln whereas 7 were decreased (table 1, second column). Three were even non-detectable in 0.6/24 gels (p2, p17A, p17B). The protein p2 for example was nicely detectable in 2.0/24 control gels but absent in the 0.6/24 gels. Then it returned to normal levels within 96 h after start of the experiment (third column). At 0.2 mM Gln, however, p2 behaved completely different: after 24 h the protein spot was slightly reduced but disappeared at 96 h. To elucidate the identity of the proteins, spots were cut out, the isolated protein was enzymatically cleaved by trypsin and the resulting peptides were analysed by PMF applying either vacuum or AP-MALDI mass spectrometry. Final identity was confirmed by PSD or low energy CID experiments generating unambiguous sequence tags. The results are shown in table 2. The spots p17A and p17B contained both the same protein (EF-Tu). In contrast, spot p22 contained GDH as well as ATP synthase alpha-chain. The MOWSE score for both proteins was above 80 and the score for a mixture was 173, which clearly confirmed the presence of the two proteins. Some proteins showed a considerable difference between the theoretical pI and the pI estimated from the spot position on the 2D gel (e.g. p22, p23, p34). It can be assumed that this was due to post-translational modifications. Unfortunately, some low abundant proteins remained non-identifiable partly due to the applied wide IPG strips. The list includes metabolic enzymes (MDH, PGDH, GDH), proteins involved in RNA processing and degradation (hnRNP F and DcpS), proteins involved in protein synthesis and degradation (EF-Tu and p45/SUG) as well as a stress protein (Hsp-4). These results show that although long-term Gln-starvation has no effect on the overall protein synthesis rate it leads to specific changes in the protein expression profile.

5. DISCUSSION

The present study reveals that Gln-utilising monocytic U937 cells activate specific adaptive mechanisms in response to Gln-starvation. The U937 cell line was derived from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma (20). These cells are well known to take up Gln (4, 21) and to utilise it for ATP synthesis (1). U937 cells react in response to Gln-starvation similarly as monocytes in respect to their overall protein synthesis and breakdown (1), their cellular hydration state (5, 6, 22) and their Hsp70 expression (4, 23). However, they are proliferating cells and differ considerably from primary monocytes. Thus, U937 cells are a well suited model system for Gln-utilising cells, however, it cannot be concluded that primary monocytes would behave identically. U937 cells show an optimal growth at 2.0 mM Gln in the medium. Lower Gln concentrations lead to a decrease in DNA synthesis rate and to a shift in the cell cycle from S-phase to G1/G0-phase (22). Thus, the optimal Gln concentration for U937 cells is far above the concentration in human blood from which these cells originally derive (plasma Gln concentration is about 0.6 mM). This might be due to the well known higher Gln utilisation rate and glutaminolysis in tumor cells (24, 25). Therefore, we regarded 2 mM Gln in the medium as normal control condition.

To expose U937 cells to Gln-starvation we cultured them in a medium with 0.6 mM or 0.2 mM Gln. The first one corresponds to the Gln concentration in plasma and was considered a mild Gln-starvation. The latter one is clearly below all ever measured pathological plasma concentrations in critically ill patients (around 0.4 mM Gln) and is therefore considered a strong Gln-starvation. Both conditions, mild and strong Gln-starvation, lead to a decrease of Gln_i below 10 % of control within the first day. A similar drop of Gln_i was recently found to occur in U937 cells already within few hours after complete withdrawal of Gln from the medium (4). This decrease was accompanied by a reduction of intracellular free glutamate. Thus, U937 cells cultured under normal conditions seems to have a very high Gln utilisation, which leads to an immediate drop of Gln_i when the extracellular source of Gln is not available anymore. However, cells cultured at 0.6 mM Gln showed in the present study an increase of Gln_i in the second 24 h to about 40 % of control. This indicates that during times of a reduced Gln supply, cells activate adaptive mechanisms in order to increase their intracellular Gln. However, these mechanisms are only able to compensate for a mild Gln-starvation. The incomplete recovery of Gln_i at 0.6 mM Gln in the medium (2,414 nmol/µg protein instead of 5,850 nmol/µg protein at 2.0 mM Gln) is accompanied by a complete restoration of the normal proliferation rate and of the normal ATP levels within four days (see Figure 1). This suggests that either a Gln_i level of about 2,500 nmol/µg protein is sufficient for these cellular functions or that the cells find a way to compensate for the still missing 3,300 nmol/µg protein Gln_i.

Cellular ATP level is tightly controlled and the AMP-activated kinase (AMPK) plays a central role in this regulation (26). Whenever the cellular ATP:ADP ratio falls, owing to a starvation that inhibits ATP production or to an increased ATP consumption, this is amplified by adenylate kinase into a much larger increase in the AMP:ATP ratio. AMP activates the AMPK by binding and phosphorylation. The present study shows that AMPK is activated by the 20 % decrease of ATP in response to a mild Gln-starvation. This activation disappears within the following three days, when the ATP level returned to normal. However, ATP levels remained stably decreased by 40 % and AMPK remained stably activated throughout the experiment in cells exposed to strong Gln-starvation. Activation of AMPK downregulates biosynthetic pathways such as fatty acid and cholesterol biosynthesis, yet switches on catabolic pathways that generate ATP, such as fatty acid oxidation,

glucose uptake and glycolysis (an interaction of AMPK with glutaminolysis is hitherto unknown). Therefore, we propose that AMPK contributes to the restoration of normal ATP levels after four days of mild Gln-starvation. However, we cannot estimate whether AMPK is involved in the regain of Gln_i under this conditions. In a recent publication, we could show that U937 cells downregulate the expression of the cell protective protein Hsp70 immediately in response to Gln-starvation by a reduced mRNA stability (4). This reduction is supposed to be responsible for the increased susceptibility of Gln-starving U937 cells to apoptosis (2). The present study revealed that Hsp70 expression decreased within 24 h in response to 0.6 mM and 0.2 mM Gln. After four days of Gln-starvation Hsp70 expression returned to normal at 0.6 mM Gln but remained decreased at 0.2 mM Gln. Thus, Hsp70 expression recovers at mild Gln-starvation and shows a similar dynamic as Gln_i, proliferation rate, ATP, and AMPK.

All these results show that U937 cells activate an adaptive mechanism in response of Gln-starvation, which enables them to overcome the lack of Gln if there is 0.6 mM Gln still available. In order to characterise the proteins involved in the adaptive response to Gln-starvation we performed a proteomic study. Cells were cultured under mild and strong Gln-starvation for 24 h and 96 h and the expression level of every single protein was compared to its expression level in control cells. This resulted in a subdivision of all proteins in four groups:, (I) transiently changed proteins, which return to normal expression after 96 h, (II) permanently changed proteins, (III) posterior changed proteins which show normal expression at 24 h but changed expression at 96 h and finally (IV) unchanged proteins. The great majority of proteins belonged to the unchanged group. Only 23 proteins where found to be affected by Gln-starvation (listed in table 1). However, it was not possible to reveal the identity of all of them. Twelve proteins were transiently down-regulated at 0.6 mM Gln: p1, p2 (Hsp-4), p14 (MDH), p17 (EF-Tu), p18, p19 (PGDH), p20, p21 (A6), p22 (GDH and ATP-S alpha), and p23 (p45/SUG). The protein spot p22 contained GDH as well as ATP-S alpha and it was not clear whether both proteins were affected similarly by Gln-starvation. Hsp-4 is a member of the Hsp70 family and exhibited a similar dynamic in its expression as Hsp70: at 0.6 mM Gln, Hsp-4 expression decreased within 24 h but regains normal values within 96 h. In contrast, at 0.2 mM Gln it decreased within 24 h and disappeared completely after 96 h. This indicates that the inhibitory effect of Gln-starvation on Hsp70 expression shown in our previous study (4) affects also other members of the heat shock protein family. However, it remains unclear whether Hsp-4 is also down-regulated by a decreased mRNA stability. Glutamate dehydrogenase (GDH) and malate dehydrogenase (MDH) are involved in glutaminolysis (27). A down-regulation of these enzymes in times of reduced availability of Gln has a high probability. The fact that this reduction was transiently at 0.6 mM Gln indicates that the regained Gln_i level in the later phase of the experiment was sufficient to reactivate glutaminolysis. However, a more detailed metabolic analysis would be necessary to proof this assumption. The enzyme 6-phosphogluconate dehydrogenase (PGDH) catalyses the third step in the pentose phosphate pathway, which is on one hand a bypass pathway for glycolysis and on the other hand provides substrate for nucleotide synthesis (28, 29). The reason for down-regulation of this enzyme remains unclear. Three proteins are transiently up-regulated by Gln-starvation: p16, p32 and p34 (PUR6). Phosphoribosyl aminoimidazole-succinocarboxamide synthase (PUR6) catalyses an important step in the synthesis of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) which is a precursor of IMP and finally all *de novo* synthesised nucleotides. AICAR is a well known strong and specific activator of AMPK (30) and has a variety of effects on cellular metabolism including increased fatty acid and glucose oxidation, enhanced GLUT2-dependent glucose uptake, suppressed IL-2 expression and stimulated IL-6 production. Two proteins were permanently changed by Gln-starvation: p26 was decreased and p30 was increased. However, it was not possible to identify these proteins due to their low abundance. Six proteins were posterior down-regulated by Gln-starvation: p5, p6 (hnRNP-F), p7, p11 (DcpS), p12, and p33. Both, the heterogeneous nuclear ribonucleoprotein F (hnRNP-F) and the scavenger mRNA decapping protein (DcpS), are involved in mRNA metabolism: hnRNP-F in mRNA processing and DcpS in mRNA degradation. The down-regulation of these proteins in the late phase of adaptation may suggest that these proteins are needed to a lesser extend in times of reduced Gln supply.

The present study revealed that Gln-utilising U937 cells activate adaptive mechanisms in response to Gln-starvation, which enable them to overcome a Gln shortage. However, when the level of available Gln falls below a critical concentration, these adaptive mechanisms are not sufficient to countervail the lack of the amino acid. We could identify a list of proteins, which are involved in this process. Although this list is incomplete and the role of all proteins is not jet understood, it gives an idea of the complex processes during Gln-starvation and might be the starting point for many future studies on Gln-starvation.

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Abbreviations: 2DE: Two-dimensional gel electrophoresis; A6: Annexin 6; AMPK: AMP activated kinase; AP-MALDI: Atmospheric pressure-MALDI; ATP: Adenosyl triphosphate; ATP-Sa: ATP Synthase a-chain; CID: Collision induced dissociation; DcpS: Scavenger mRNA decapping enzyme; DTT: Dithiotreitol; EF-Tu: Elongation factor Tu; FCS: Fetal calf serum; FIV: Fluorescence intensity volume; GDH: Glutamate dehydrogenase 1; Gln: Glutamine; GSH: Glutathione; hnRNP F: Heterogeneous nuclear ribonucleoprotein F; Hsp-4: Heat shock 70 kDa protein 4; Hsp70: 70kDa heat shock protein; IEF: Isoelectric focusing; MALDI: Matrix-Assisted Laser Desorption/Ionization; MDH: Malate dehydrogenase; MS: Mass spectrometry; OPA: o-Phtalaldehyde; p45/SUG: 26S protease regulatory subunit 8; PBS: Phosphate buffered saline; PDF: Pulsed dynamic focusing; PGDH: 6-Phosphogluconate dehydrogenase; PUR6 : Phospho ribosyl amino imidazole-succino carboxamide synthase; SAP 61: Splicing factor 3A subunit 3; SDS-PAGE: Sodium dodecyl sulfate polyacryl amide gel electrophoresis; TCA: Tricloacetic acid; TNF-alpha: Tumor necrosis factor alpha; TOF: Time of flight

Key Words: Glutamine, Amino acid starvation, Starvation response, Stress proteins, Proteomics

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