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Research paper

Activation of the antioxidant response in methionine deprived human cells results in an HSF1-independent increase in HSPA1A mRNA levels

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ABSTRACT

In cells starved for leucine, lysine or glutamine heat shock factor 1 (HSF1) is inactivated and the level of the transcripts of the HSF1 target genes HSPA1A (Hsp70) and DNAJB1 (Hsp40) drops. We show here that in HEK293 cells deprived of methionine HSF1 was similarly inactivated but that the level of HSPA1A and DNAJB1 mRNA increased. This increase was also seen in cells expressing a dominant negative HSF1 mutant (HSF379 or HSF1-K80Q), confirming that the increase is HSF1 independent. The antioxidant N-acetylcysteine completely inhibited the increase in HSPA1A and DNAJB1 mRNA levels upon methionine starvation, indicating that this increase is a response to oxidative stress resulting from a lack of methionine. Cells starved for methionine contained higher levels of c-Fos and FosB mRNA, but knockdown of these transcription factors had no effect on the HSPA1A or DNAJB1 mRNA level. Knockdown of NRF2 mRNA resulted in the inhibition of the increase in the HSPA1A mRNA, but not the DNAJB1 mRNA, level in methionine starved cells. We conclude that methionine deprivation results in both the amino acid deprivation response and an antioxidant response mediated at least in part by NRF2. This antioxidant response includes an HSF1 independent increase in the levels of HSPA1A and DNAJB1 mRNA.

1. Introduction

Amino acids are the building blocks of proteins and can also serve as intermediates in metabolism. The amino acid availability is closely monitored [1]. When cells sense a lack of one or more amino acids the amino acid response is mounted. Upon accumulation of uncharged tRNAs the general control non-derepressible 2 (GCN2) kinase is activated [2] and subsequently eukaryotic translation initiation factor 2α (eIF2α) is phosphorylated. eIF2α phosphorylation then leads to the inhibition of the global protein synthesis and the selective translation of some mRNAs, e.g. ATF4 mRNA (reviewed in Ref. [3]). ATF4 is an important player in the amino acid response: most amino acid responsive genes are ATF4 targets of which asparagine synthetase (ASNS) is the most widely studied one [4,5]. Petti et al. [6] showed that in yeast methionine starvation differs from starvation for other amino acids in that survival was substantially higher compared to for example leucine starvation. Furthermore, methionine has been shown to have a unique effect on fecundity in *Drosophila* upon dietary restriction, a restriction in food intake that does not lead to malnutrition. Grandison et al. [7] described a decrease in fecundity upon reduced food intake, and adding back methionine alone was sufficient to increase fecundity to the same level as did full feeding. Adding back other amino acids did not show this effect. These data thus suggest that starvation for methionine does not equal starvation for other amino acids.

Next to its importance in protein synthesis, the essential amino acid methionine is also involved in the transsulfuration pathway, a pathway in which methionine is converted via the formation of S-adenosylmethionine (SAM) into homocysteine and subsequently cysteine [8]. Cysteine availability is important for the synthesis of glutathione, a molecule that has been shown to have a strong antioxidative effect. A lack of methionine might thus have an effect on the formation of glutathione and can thereby affect the oxidative status of the cell. In the literature conflicting results about methionine deprivation and the antioxidant response are described. Erdmann et al. [9] showed that addition of L-methionine reduced free radical formation in endothelial cells through the

Abbreviations: GCN2, general non-derepressible 2; ATF, activating transcription factor; ASNS, asparagine synthetase; SAM, S-adenosylmethionine; HMOX1, heme oxygenase 1; GSTP, glutathione S-transferase P; HSF1, heat shock factor 1; HSE, heat shock element; NAC, N-acetylcysteine; NRF2, nuclear factor (erythroid-derived 2)-related factor 2; ARE, antioxidant response element; TPA, 12-*O*-tetradecanoyl phorbol13-acetate; TRE, TPA-responsive element; KEAP1, Kelch-like ECH associated protein 1; EpRE, electrophile-responsive element.

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induction of heme oxygenase 1 (HMOX1). On the other hand it was described that methionine supplementation increases mitochondrial ROS production and mitochondrial DNA oxidative damage in rat liver mitochondria [10] and vice versa that methionine restriction decreases mitochondrial ROS generation and oxidative damage to mitochondrial DNA and proteins, indicating the activation of the antioxidant response [11,12]. Recently, Lin et al. [13] demonstrated that the increased synthesis of glutathione S-transferase P (GSTP) in methionine restricted rat hepatocytes is due to activation of the transcription factor NRF2, a factor involved in the antioxidant response.

We have previously shown that upon starvation for leucine, lysine or glutamine the transcription factor heat shock factor 1 (HSF1), which regulates the proteotoxic stress response, is inactivated and that the mRNA levels of the HSF1 target genes HSPA1A, DNAJB1 and HSP90AA1 levels are strongly decreased [14]. The complex cellular response to methionine starvation made us wonder whether HSF1 is also inactivated in methionine starved cells. We show here that HSF1 indeed also loses its DNA binding affinity in methionine starved cells, but that, unexpectedly and in contrast to what is found during starvation for other amino acids, HSPA1A and DNAJB1 mRNA levels do increase. This increase was not dependent on HSF1 but, at least for HSPA1A mRNA, on NRF2. These data show that in methionine starved cells an antioxidant response is superimposed on the amino acid response.

2. Materials and methods

2.1. Tissue culture

T-REx HEK293-pcDNA5, HEK-HSF379 (dnHSF1) and HEK-HSF1 K80Q cell lines were generated as described before [14,15]. The cells were cultured at 37 °C/5% CO_2 in high glucose DMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Blasticidin (1.65 μg/ml; Invitrogen) and 100 μg/ml hygromycin were also added to the culture medium during maintenance of the cell lines, but were omitted during experiments. For amino acid starvation experiments cells were washed with PBS and subsequently DMEM/F12 medium (Sigma) with or without methionine or leucine, supplemented with 10% dialyzed fetal calf serum, was added for the indicated times.

2.2. RNA isolation and reverse transcription

Total RNA was isolated using Trizol (Invitrogen). 1 μg of RNA was treated with DNaseI (Amplification grade; RNase-free; Invitrogen). Subsequently, 5 mM $MgCl_2$, RT-buffer, 1 mM dNTPs, 18.75 units AMV reverse transcriptase, 20 units RNase inhibitors and 1.25 μM oligo(dT) were added to a total volume of 20 μl. Reverse transcription was performed for 10 min at 25 °C, 60 min at 42 °C and 5 min at 95 °C. For QPCR analysis, cDNA was 10-fold diluted.

2.3. Chromatin immunoprecipitation

T-REx HEK293-pcDNA5 cells were cultured for 24 h in the presence or absence of methionine. Chromatin immunoprecipitation was performed as described in Ref. [16], except that cells were crosslinked for 15 min with 1% formaldehyde. After quenching with 125 mM glycine, cells were washed twice with ice cold PBS and resuspended in ice cold lysis buffer (50 mM HEPES·KOH pH 7.6, 140 mM NaCl, 1 mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.1% NaDOC and 1× protease inhibitor cocktail). Antibodies used for ChIP were rabbit polyclonal ATF4 antibody (sc-200; Santa Cruz) and rabbit polyclonal HSF1 antibody (SPA-901; Stressgen). ChIP samples were analyzed by QPCR with the primer sets listed in Supplementary data Table S1.

2.4. Electrophoretic mobility shift assay

HEK293 cells were cultured for 24 h in the presence or absence of methionine. Cells were immediately harvested and nuclear extracts were prepared using NE-per nuclear and cytoplasmic reagents (Pierce). Extracts were aliquoted and stored at −80 °C. Oligonucleotide probes were end-labeled with ^{32}P. The sequences of the oligonucleotides used in EMSA are listed in Supplementary data Table S1. The EMSA protocol was adapted from Refs. [17,18]. A mixture containing 5 μg nuclear extract and 3 μg poly dIdC in binding buffer [20 mM HEPES pH 7.9, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 4% (v/v) Ficoll, 1× PhosSTOP (Roche)] was incubated for 20 min on ice. 0.01 pmol radiolabeled oligonucleotide was added and the samples were incubated for 20 min at room temperature. DNA–protein complexes were separated on a pre-run 4% polyacrylamide gel in 0.25× TBE with recirculation of the buffer. The gel was dried and signals were visualized using a PhosphorImager.

2.5. RNA interference

The control siRNA against luciferase (5′-CGUACGCGGAAUACUUCGAdTdT-3′), NRF2 siRNA (5′-CAGCAUGCUACGUGAUGAAdT dT-3′) and NRF2 siRNA#2 (5′-CCAGUGGAUCUGCCAACUAdT dT-3′) were purchased from Eurogentec. C-Fos (sc-29221) and FosB (sc-35403) siRNAs were purchased from Santa Cruz Biotechnology. HEK293 cells were cultured in 6-well plates and transfected with 50 nM siRNA using oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. 48 h after transfection cells were re-transfected as described above. Medium was changed to DME/F12 with or without methionine 24 h after re-transfection and cells were harvested 24 h later.

2.6. Quantitative real-time PCR

Quantitative real-time PCR was performed using the StepOnePlus™ Real-Time PCR System with *Power* SYBR® Green PCR Master mix (Applied Biosystems) using the following amplification protocol: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Per reaction 3 μl of diluted cDNA or ChIP material was used and the DNA was amplified using primers for the sequences of interest, listed in Supplementary data Table S1. Two-tailed Student's *t* tests were performed to calculate the significance of the data.

3. Results and discussion

3.1. Increased HSPA1A and DNAJB1 mRNA levels in methionine deprived HEK293 cells

In a previous study we described that leucine, lysine or glutamine deprivation results in the inactivation of HSF1 and a concomitant decrease in heat shock protein mRNA levels [14]. To determine whether methionine deprivation has a similar effect, we measured HSPA1A and DNAJB1 mRNA levels in methionine starved HEK293 cells and found these, in contrast with the effect of leucine, lysine and glutamine starvation, to be increased (Fig. 1A). Already within 4 h of methionine starvation an increase in HSPA1A mRNA was seen; the maximal level was reached within 8 h (Fig. 1B). The mRNA level of DNAJB1 increased only later (Fig. 1C). Note that, although the increase in HSPA1A mRNA level upon methionine deprivation was consistently found, the extent of change in the HSPA1A mRNA levels varied between 3- and 30-fold between