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Homocysteine thiolactone affects protein ubiquitination in yeast

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The formation of homocysteine thiolactone (HcyTl) from homocysteine occurs in all examined so far organisms including bacteria, yeast, and humans. Protein *N*-homocysteinylation at the ε-amino group of lysine is an adverse result of HcyTl accumulation. Since tagging of proteins by ubiquitination before their proteasomal degradation takes place at the same residue, we wondered how *N*-homocysteinylation may affect the ubiquitination of proteins. We used different yeast strains carrying mutations in genes involved in the homocysteine metabolism. We found positive correlation between the concentration of endogenous HcyTl and the concentration of ubiquitinated proteins. This suggests that *N*-homocysteinylation of proteins apparently does not preclude but rather promotes their decomposition.

Key words: homocysteine, homocysteine thiolactone

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INTRODUCTION

Homocysteine (Hcy) is a non protein amino acid synthesized from methionine (*via* adenosylmethionine and adenosylhomocysteine). Since Hcy is similar to methionine it is accidentally activated by methionyl-tRNA synthetase and in error editing reaction is converted to homocysteine thiolactone (HcyTl) (Jakubowski & Fersht, 1981; Jakubowski, 1990; 1991; Jakubowski & Goldman, 1993). HcyTl contains high energy thioester bound and uses that stored energy for the formation of isopeptide bond between the carboxyl group of homocysteine and ε-amino group of lysine in a reaction called *N*-homocysteinylation (Jakubowski, 1999; Jakubowski *et al.,* 2000). Thus, homocysteine is not built into protein directly during the translation process, but it can be incorporated into protein *via N*-homocysteinylation (Fig. 1). *N*-Homocysteinylated proteins have been found in microorganisms and mammals including humans (Jakubowski *et al.,* 2000; Perla-Kajan *et al.,* 2008; Jakubowski *et al.,* 2009; Sikora & Jakubowski, 2009). Human serum contains a significant concentration of protein-bound homocysteine; 1 molecule of homocysteine per 1000 or 1670 molecules of methionine is present in hemoglobin or albumin, respectively (Jakubowski, 2002). *N*-Homocysteinylation causes severe consequences, i.e., leads to loss of enzymatic activity, formation of amyloid-like structures (Paoli *et al.,* 2010), and multimerisation of extensively modified proteins (Jakubowski, 1999). Protein *N*-homocysteinylation is considered as one of the mechanisms that are responsible for homocysteine toxicity (Jakubowski, 1999; 2002; Jakubowski *et al.,* 2009; Zabczyk *et al.,* 2011).

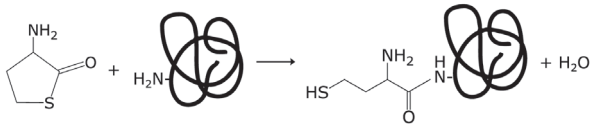

Figure 1. Homocysteine thiolactone spontaneously *N*-homocysteinylates ε-amino groups of lysine residue in proteins (Jakubowski, 1997).

Organisms developed several mechanisms against HcyTl toxicity. Three enzymes hydrolyzing homocysteine thiolactone have been found in mammals: serum paraoxonase (Jakubowski, 2000), intracellular bleomycin hydrolase (Zimny *et al.,* 2006), and mitochondrial thiolactonase (Zimny *et al.*, 2011). Bleomycin hydrolase is highly conserved and widely distributed in yeast and different vertebrates (Enenkel & Wolf, 1993; Joshua-Tor *et al.,* 1995; O'Farrell *et al.,* 1999; Zimny *et al.,* 2006). Large amounts of HcyTl are excreted with urine (Chwatko & Jakubowski, 2005). Finally, autoantibodies against *N*-homocysteinylated proteins have been found in humans (Undas *et al.,* 2004). In human serum homocysteine is present in different forms: as a free homocysteine (100 nM), oxidized as a homocystine and mixed disulfide with cysteine and albumin (9.3 μM), protein bound (15.5 μM), and as a free HcyTl (0–35 nM) (Jakubowski, 2006).

Concentration of HcyTl strictly depends on the concentration of homocysteine, what is affected mainly by the concentration of methionine and genotype. Organisms lacking activity involved in remetylation or transsulfuration of Hcy accumulate homocysteine and produce high concentration of homocysteine thiolactone (Jakubowski, 1990; 1991; 1997). Consequently, the concentration of *N*-homocysteinylated proteins also rises (Jakubowski, 1999; Jakubowski *et al.,* 2000; Jakubowski *et al.,* 2009). *N*-Hcy-hemoglobin is digested by mice liver extracts and distinctive product of this degradation, the isopeptide *N*-Hcy-Lys, has been found in human and mice plasma (Glowacki *et al.,* 2010). Therefore, the question arises: may enhanced protein *N*-homocysteinylation induce protein turnover, would be reflected in enhanced protein ubiquitination? Here we describe how homocysteine thiolactone affects protein ubiquitination of different yeast strains carrying mutations of genes encoding enzymes involved in homocysteine metabolism.

MATERIALS AND METHODS

Chemicals. Essential laboratory chemicals and yeast media components including D,L-homocysteine were obtained from Sigma Aldrich (St. Louis, MO, USA), anti-ubiquitin antibodies (rabbit polyclonal antibody cat. no:

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Abbreviations: Hcy, homocysteine; HcyTl, homocysteine thiolactone

Table 1. Yeast strains used in this study

	Strain name and genotype	Description of mutation	Metabolic effect of mutation	Source
1	ABJ3D MAT α *met6 mes1*	Inactivation of methionine--tRNA synthetase	Formation of homocysteine thiolactone disabled	H. Jakubowski (Jakubowski, 1991)
2	ABJ-6 MAT a *cys2,4, ura3*	Inactivation of homocysteine transsulfuration pathway	Produces high concentration of homocysteine thiolactone	H. Jakubowski (Jakubowski, 1991)
3	HWY22 MAT a *his3-1 leu2-0 met15-0 ura3-0*/pYES2	–	Wild type control for HWY22	D. Ramotar (Wang & Ramotar, 2002)
4	HWY24 MAT a *his3-1 leu2-0 met15-0 ura3-0 blh1Δ* ::KanMX/pYES2	Inactivation of thiolactonase/ bleomycin hydrolase	Produces higher concentration of homocysteine thiolactone than wild type HWY22	D. Ramotar (Wang & Ramotar, 2002)
5	S288C MAT α wt-gal	–	Wild type	H. Jakubowski (Jakubowski, 1991)
6	XJB3-1B MAT α *met6*	Inactivation of homocysteine remethylation pathway	Produces the highest concentration of homocysteine thiolactone among known strains	H. Jakubowski (Jakubowski, 1991)

3933S, Lot. 2) and chemiluminescent substrate (Lumi-Glo) for horseradish peroxidase (HRP) were obtained from Cell Signaling (Beverly, MA, USA), and secondary antibody conjugated with HRP was obtained from Jacson Immunoresearch Laboratories (West Grove, PA, USA),

Yeast strains. The yeast strains used in this work, listed in Table 1, were kindly provided by Dr. H. Jakubowski.

Yeast cultures. Single colonies of yeast strains from YPD plates were placed in liquid minimal media and incubated at 30°C until stationary phase was reached. After the passage, yeast were cultured overnight on minimal liquid media plus auxotrophic requirements. After that time, OD_{600} of different strains reaches different values (from 0.3 to 1.2) which depends on the genotype. Cultures of each strain were divided in two, one part was further incubated with Hcy (10 μmol/10^8 cells) and the other part was incubated as a control without the addition of Hcy. After 3 hours of incubation OD_{600} of cultures was measured and equal number of cells from each culture was harvested by centrifugation. Media were collected and subjected to the analysis of HcyTl concentration by HPLC and the cells were washed with water and frozen at –20°C.

HPLC analysis. The concentration of HcyTl in media was quantified on JASCO HPLC system with diode-array detector using a method described by Jakubowski (Jakubowski, 2002). Briefly, 10 μl of medium containing up to 1 μmol of homocysteine thiolactone was injected on the cation exchange column (polysulfoethyl aspartamide, PolyLC, 200 Å, 5μ, 35 × 2.1 mm). The column was washed isocratically with 10 mM sodium phosphate buffer pH 6.6 containing 150 mM NaCl at 0.36 ml/min flow rate. The presence of HcyTl was monitored at 237 nm. At those conditions HcyTl was eluted at 2 min while the remaining compounds of media did not retain on column and were eluted at 1 min.

Protein extraction. Disruption of yeast and protein extraction were carried out according to Kushnirov's method (Kushnirov, 2000). Equal portions of centrifuged yeast (2.5 OD_{600}) were resuspended in 200 μl of 0.1M NaOH, incubated at room temperature, pelleted, resuspended in 50 μl of SDS sample buffer (0.06 M Tris/HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol), boiled for 3 minutes and pelleted again. Typical extracts contained protein in concentration 2.7 ± 0.3 mg/ml.

Protein concentration. Protein concentration was measured by the turbidimetric micromethod of determination of proteins with tannin (Mejbaum-Katzenellenbogen, 1955) adapted to microplates.

Dot blot analysis. Yeast extracts (2.5 μg of protein per well) were diluted in TBST (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1 % Tween-20) and applied on nitrocellulose membrane (0.45 μm, Schleicher and Schuell) using Bio-dot apparatus (Bio-Rad). The membrane was blocked with 3% bovine serum albumin dissolved in TBST. After 1 h of blocking, 10 μl of primary antibodies (anti-ubiquitin) were added to the solution and incubated for 90 minutes. Next, the membrane was washed with TBST (3 times for 10 minutes), 10 μl of secondary antibodies in TBST were added and incubated for 1 h followed by 3 times wash with TBST. Subsequently, the membrane was placed on foil and 1 ml of Lumi-Glow was poured on the membrane's surface and after one minute of incubation the membrane was covered with foil and placed in an exposure cassette with Kodak X-ray film. After 3 minutes of exposition, the film was developed and analyzed on G-box with GeneTools software. Samples from each series of experiments were analyzed simultaneously. Figure 2 shows representative dot blot for the ubiquitinated protein assay.

RESULTS AND DISCUSSION

Homocysteine added to yeast culture enters the cells and is further metabolized. One of metabolites formed from homocysteine is homocysteine thiolactone that can easily diffuse through cellular membranes and therefore its concentration can be measured in the media (Jakubowski, 1991; 2002). High concentration of homo-

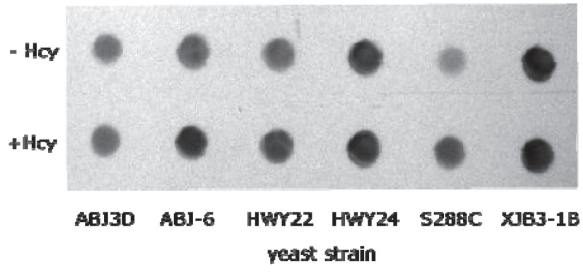

Figure 2. Representative dot blot for the ubiquitinated protein assay.

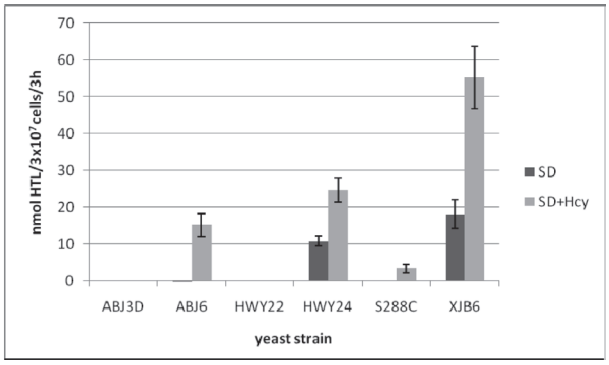

Figure 3. Average homocysteine thiolactone (HcyTl) concentration in the minimal growth media (SD) of different yeast strains cultured with or without homocysteine (Hcy) (10 µmol/10⁸ cells, n=3).
Wild type yeast (HWY22 and S288C) as well as *met6 mes1* strain (ABJ3D) produced the lowest levels of homocysteine thiolactone while those with impaired Hcy metabolism (ABJ6 *cys2,4 ura3*, XJB3-1B *met6*) and deactivated thiolactonase (HWY24 *blh1Δ* ::KanMX/pYES2) formed high concentrations of HcyTl.

cysteine is detrimental for yeast cells and mechanism of this toxicity is driven by HcyTl (Zimny *et al.,* 2006). Adverse protein *N*-homocysteinylation is caused by HcyTl and its rate strictly depends on homocysteine thiolactone concentration (Jakubowski, 1999). In this study yeast were grown on minimal media supplemented with homocysteine to provoke intracellular formation of HcyTl and *N*-homocysteinylation of proteins. After 3h of incubation with Hcy, different yeast strains released different quantities of homocysteine thiolactone, and the value depended on their genotypes. Our findings are consistent with previously published observations (Jakubowski 1991; 2002; Zimny *et al.,* 2006). In media from control cultures (without Hcy), the formation of HcyTl was considerably lower (Fig. 3).

Wild type strains (S288C and HWY22) exhibited low levels of ubiquitinated proteins and this was independent of the presence of Hcy in the growth media. Similarly, strain that is unable to convert Hcy to HcyTl due to *mes1* mutation (ABJ3D) showed low and homocysteine independent level of ubiquitinated proteins. In contrary, the *cys2,4* (ABJ6), *met6* (XJB3-1B) and *blh1* (HWY24) mutants exhibited high concentration of ubiquitinated proteins (Fig. 4). Concentration of ubiquitinated proteins in yeast extracts was positively correlated with the concentration of HcyTl excreted by cells. Pearson's correlation for those features was $r = 0.81$ and this correlation is significant at the 0.01 level ($p = 0.001$; $n = 18$).

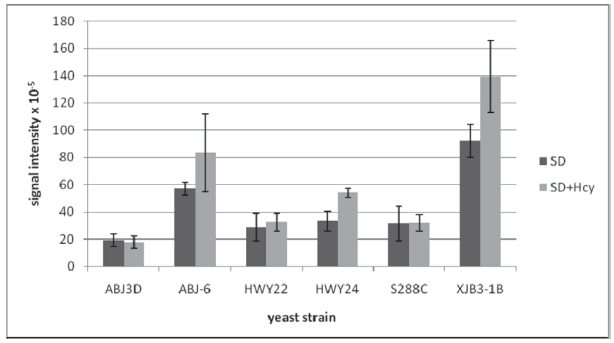

Figure 4. Average levels of ubiquitinated proteins in the extracts from different yeast strains with (SD+Hcy, n=3) and without homocysteine in the medium (SD, n=3).

Both *N*-homocysteinylation and ubiquitination take place at the ε-amino group of lysine residue. However, protein ubiquitination is much more prevalent than *N*-homocysteinylation. Proteins have usually several lysine residues and the results of our work show that *N*-homocysteinylation apparently does not compete with ubiquitination. Human albumin contains 63 lysine residues and 7 of them (Lys-4, Lys-12, Lys-137, Lys-159, Lys-205, Lys-212 and Lys-525) are *N*-homocysteinylated *in vitro* while only 3 of them (Lys-137, Lys-212 and Lys-525) are *N*-homocysteinylated *in vivo* (Marczak *et al.,* 2011). Computer analysis of potential ubiquitination sites in human albumin reveals that 17 lysine residues are prone to ubiquitination but only one (Lys-4) of them can be also *N*-homocysteinylated (Radivojac *et al.,* 2010). Moreover, free ε-amino groups of lysine not always are crucial for marking proteins for proteosomal degradation. Selective blocking of ε-amino groups of lysozyme and albumin did not prevent them from ubiquitin-dependent degradation, and the same proteins with blocked α-amino groups were not susceptible to degradation (Hershko *et al.,* 1984).

Taken together, data from these experiments suggest that protein *N*-homocysteinylation may either directly or indirectly (i.e., by changing protein conformation and/or causing protein aggregation) mark protein for ubiquitination. The enhanced protein *N*-homocysteinylation is likely to induce protein turnover, which is reflected in promoted protein ubiquitination.

Whereas the extracts from ABJ3D strain which does not produce HcyTl practically did not contain ubiquitinated proteins, regardless of the presence or absence of homocysteine in the media, the extracts from other strains contained different amounts of ubiquitinated proteins and their concentration increased with the increase of HcyTl concentration. Therefore it was rather not homocysteine itself but homocysteine thiolactone that affected the level of ubiquitinated proteins in the yeasts.

CONCLUSIONS

The formation of HcyTl up regulates protein ubiqutination. The reason for the intensification of labeling proteins for proteosomal degradation is probably recognition of *N*-homocysteinylated proteins as damaged ones.

It is homocysteine thiolactone not homocysteine itself that affects the concentration of ubiquitinated proteins

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