Homocysteine- and cysteine-mediated growth defect is not associated with induction of oxidative stress response genes in yeast

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Intracellular thiols like cysteine, homocysteine and glutathione play a critical role in the regulation of important cellular processes. Alteration of intracellular thiol concentration results in many diseased states; for instance, elevated levels of homocysteine are considered to be an independent risk factor for cardiovascular disease. Yeast has proved to be an excellent model system for studying many human diseases since it carries homologues of nearly 40 % of human disease genes and many fundamental pathways are highly conserved between the two organisms. In the present study, we demonstrate that cysteine and homocysteine, but not glutathione, inhibit yeast growth in a concentration-dependent manner. Using deletion strains (*str2Δ* and *str4Δ*) we show that cysteine and homocysteine independently inhibit yeast growth. Transcriptional profiling of yeast treated with cysteine and homocysteine revealed that genes coding for antioxidant enzymes like glutathione peroxidase, catalase and superoxide dismutase were down-regulated. Furthermore, transcriptional response to homocysteine did not show any similarity to the response to H₂O₂.

We also failed to detect induction of reactive oxygen species in homocysteine- and cysteine-treated cells, using fluorogenic probes. These results indicate that homocysteine- and cysteine-induced growth defect is not due to the oxidative stress. However, we found an increase in the expression of *KAR2* (karyogamy 2) gene, a well-known marker of ER (endoplasmic reticulum) stress and also observed *HAC1* cleavage in homocysteine- and cysteine-treated cells, which indicates that homocysteine- and cysteine-mediated growth defect may probably be attributed to ER stress. Transcriptional profiling also revealed that genes involved in one-carbon metabolism, glycolysis and serine biosynthesis were up-regulated on exogenous addition of cysteine and homocysteine, suggesting that cells try to reduce the intracellular concentration of thiols by up-regulating the genes involved in their metabolism.

Key words: cysteine, glutathione, homocysteine, reactive oxygen species, thiol, yeast.

INTRODUCTION

Intracellular concentration of thiols is highly regulated and any alteration in their concentrations can potentially lead to severe disease states. Among the biologically important thiols, homocysteine, a thiol amino acid formed during the metabolism of methionine, has received increasing attention during the past decade as elevated levels of homocysteine have been implicated as an independent risk factor for cardiovascular disease [1,2]. High levels of homocysteine have also been associated with various other diseases and/or clinical conditions including Alzheimer's disease [3], neural tube defects [4], schizophrenia [5], end-stage renal disease [6], osteoporosis [7] and Type II (non-insulin-dependent) diabetes [8]. Some recent reports also suggest that elevated levels of cysteine might be associated with cardiovascular diseases [9,10]. Cysteine has also been shown to have cytotoxic effects [11]. Elevated levels of glutathione, however, play a protective role by scavenging free radicals and decreasing the intracellular oxidative stress [12].

Both homocysteine and cysteine are produced intracellularly during the metabolism of methionine [13]. Homocysteine is a key branch point intermediate in the ubiquitous methionine cycle, the function of which is to generate one-carbon methyl groups for transmethylation reactions that are very much essential to all life forms [13]. Although homocysteine has been associated with several diseases, the mechanism(s) underlying the deleterious effects of homocysteine has not yet been completely elucidated. It is generally proposed that, in circulation, homocysteine under-

goes rapid autoxidation in the presence of metal ions to form reactive oxygen species, thus leading to endothelial dysfunction [14]. However, several recent reports indicate that although cysteine in plasma undergoes rapid metal-catalysed oxidation, homocysteine is mainly oxidized by albumin via thiol disulphide exchange reaction where the generation of peroxides is minimal [15,16]. The deleterious effect of homocysteine might also result from the ability of homocysteine to potentially disrupt critical protein disulphide bonds, thereby altering the structure and/or function of proteins [17–19]. Furthermore, increase in the concentration of homocysteine results in elevated concentration of SAH (S-adenosyl homocysteine), which is an inhibitor for many methyl transferases including DNA methyl transferase [20]. This might result in the hypomethylation of genes, leading to altered gene expression. Some recent reports do indicate that hyperhomocysteinaemic patients have an altered global methylation pattern [21].

To better understand how elevated levels of homocysteine and/or cysteine might impact cellular physiology and pathology, we have used the yeast *Saccharomyces cerevisiae* as a model system to study the effect of exogenously added thiols, i.e. homocysteine, cysteine and glutathione. *S. cerevisiae* shows considerable similarity to higher systems in cellular organization and function and has been used as a model system for studying many phenomena of relevance to human biology at the molecular level [22,23]. We as well as others have previously used gene expression profiling in wild-type yeast cells and knockout mutants after treatment with environmental perturbations and nutritional

Abbreviations used: DCFH-DA, 2',7'-dichlorofluorescein diacetate; DTT, dithiothreitol; ER, endoplasmic reticulum; GRP78, glucose-regulated protein, 78 kDa; HPLC-FD, HPLC equipped with a fluorescence detector; RT, reverse transcriptase; SAH, S-adenosyl homocysteine.

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changes to study transcriptional regulation [24–26]. The basic molecular machinery participating in fundamental cellular processes like replication and transcription is conserved from yeast to human. The methionine metabolism pathway in *S. cerevisiae* is similar to that in human with some minor variations. While previous studies have focused on the role of *S*-adenosyl methionine and SAH in transcriptional regulation of genes involved in sulphur metabolism [27], the effect of thiols on yeast growth and global gene expression has not been studied.

In the present study, we demonstrate that both cysteine and homocysteine affect the growth of yeast and also show that the gene expression pattern of yeast is altered after exogenous addition of homocysteine and cysteine. Addition of these thiols down-regulated genes coding for antioxidant enzymes, while genes coding for enzymes that metabolize homocysteine were up-regulated.

MATERIALS AND METHODS

Materials

DL-Homocysteine, L-homocysteine thiolactone, cysteine and mono-bromobimane were purchased from Sigma (St. Louis, MO, U.S.A.). The constituents of yeast media including yeast extract, peptone, dextrose and the amino acids were purchased from HiMedia (India). All other chemicals used were of analytical grade.

Yeast strains, media and growth conditions

The wild-type *S. cerevisiae* strain used in the present study is BY4742 (MAT α his3 Δ -1 leu2 Δ -0 lys2 Δ -0 ura3 Δ -0). Deletion strains *str2* Δ and *str4* Δ were procured from the EUROSCARF (European *S. cerevisiae* archive for functional analysis) deletion collection (EUROSCARF, Institute of Microbiology, Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany). Rich media for culturing yeast (YPD medium) contained 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose, and for solid media, 2% (w/v) agar was added to YPD liquid media. Synthetic minimal media contained 2% (w/v) glucose, 0.17% yeast nitrogen base without amino acids, 0.5% NH₄Cl supplemented with the following amino acids: adenine, 40 μ g/ml; L-arginine (HCl), 20 μ g/ml; L-aspartic acid, 100 μ g/ml; L-glutamic acid (monosodium salt), 100 μ g/ml; L-histidine, 20 μ g/ml; L-leucine, 60 μ g/ml; L-lysine (mono-HCl), 30 μ g/ml; L-phenylalanine, 50 μ g/ml; L-threonine, 200 μ g/ml; L-tryptophan, 40 μ g/ml; L-tyrosine, 30 μ g/ml; L-valine, 150 μ g/ml; and uracil, 20 μ g/ml. To overcome the cysteine auxotrophy in *str4* Δ strains, 500 μ M of glutathione was added.

Growth studies

Precultures were prepared by growing a 5 ml yeast culture in rich medium for 12–14 h. These cultures, grown overnight, were washed three times with sterile water and then resuspended in 1 ml of sterile water. The resuspended cells were inoculated in 20 ml of synthetic minimal media supplemented with amino acids as mentioned above, to an A_{600} (absorbance) of 0.1. To study the effect of exogenously added thiols on yeast growth, various concentrations of homocysteine, cysteine or glutathione were added (from freshly prepared stock solutions) and cells were grown on a rotary shaker at room temperature (28 °C) at 150 rev./min. In all the studies, unless otherwise mentioned, DL-homocysteine was used. Yeast growth was monitored by withdrawing aliquots from flasks at different time points and measuring the turbidity

(attenuance) (at 600 nm) using a spectrophotometer (PerkinElmer Lambda bio 20).

Determination of intracellular homocysteine and cysteine levels

Yeast cells with or without exogenously added thiols were grown in synthetic minimal media for a defined time period. The number of cells in the culture was calculated from the A_{600} (1 absorbance unit = 3×10^7 cells). Cells were then harvested by centrifugation and washed three times with sterile water containing 3 mM DTT (dithiothreitol) to remove homocysteine/cysteine that may potentially bind to the cell surface. This was followed by washing the pellet three times with sterile water to remove the DTT. The pellet was resuspended in 0.1 ml of water and lysed by 10 rounds of vortex-mixing with glass beads for 1 min each. The suspension was then centrifuged at 14000 *g* for 10 min at room temperature and the level of thiols was determined in the supernatant using HPLC-FD (HPLC equipped with a fluorescence detector) as described in [28]. Briefly, 0.065 ml of yeast lysate was treated with 0.035 ml of 1.43 M sodium borohydride in 0.10 M sodium hydroxide (to reduce oxidized thiols) followed by the addition of 0.035 ml of 1.0 M HCl. To this, 0.05 ml of 7 mM monobromobimane in 5 mM sodium EDTA (pH 7.0) was added (to conjugate the reduced thiols with the fluorophore) and the solution was incubated at 42 °C for 12–15 min. Intracellular proteins were then precipitated by the addition of 0.050 ml of 1.5 M HClO₄ followed by centrifugation at 14000 *g* for 5 min. The supernatant was then transferred to injector vials for automated HPLC analysis. HPLC measurements were done by using Agilent 1100 using reverse-phase C18 column (5 μ M bead size; 4.6 mm $\times$ 150 mm from Phenomenex, U.S.A.). Standard curves were generated with known amounts of homocysteine and cysteine to calculate the concentration of these thiols in the yeast lysate.

Determination of free reduced thiols in the medium

To check the levels of free reduced thiols in the medium during the growth curve experiments, 0.05 ml aliquots were withdrawn from the media at each time point and diluted to 0.5 ml with water. The solution was then centrifuged and 0.1 ml of the supernatant was added to 0.05 ml of monobromobimane (7 mM). The solution was then incubated at 42 °C for 12–15 min and then analysed using HPLC-FD. The concentrations of free reduced thiols were determined using standard curves obtained by treating known concentrations of free reduced cysteine and homocysteine.

Detection of reactive oxygen species

Intracellular redox levels were measured by fluorimetry using the fluorescent dye DCFH-DA (2',7'-dichlorofluorescein diacetate). Cells were grown in minimal media for 12 h with or without the exogenous addition of homocysteine, cysteine or glutathione. To a set of untreated flasks, 1 mM H₂O₂ was added 1 h before harvesting the cells. Cells were collected by centrifugation, 12 h after the exogenous addition of thiols, and then washed three times with PBS. Cells were resuspended in PBS with 10 μ M DCFH-DA (Sigma) and incubated at 28 °C for 1 h. Cells were collected, washed three times with PBS and lysed using glass beads. The fluorescence intensity of an aliquot of the lysate was measured on a Fluoromax3, SPEX spectrofluorimeter. The dye was excited at 488 nm and emission was monitored from 520 nm. The protein content of the lysate was determined using bicinchoninic acid (Sigma). The observed intensity of each sample was normalized with the protein content of cell lysate.

RNA isolation and Northern-blot analysis

Total RNA was isolated using glass bead lysis followed by hot phenol method [29] and Northern-blot analyses were done as described in [29]. Briefly, RNA was electrophoresed on 1.2 % (v/v) denaturing formaldehyde gel and blotted on to nylon membrane (Hybond-N+; Amersham, U.S.A.) and immobilized by UV cross-linking. Prehybridization and hybridization were performed at 65 °C in Church buffer containing 7 % (w/v) SDS and 0.2 M sodium phosphate buffer (pH 7.2; monobasic sodium dihydrogen orthophosphate and dibasic disodium hydrogen orthophosphate dehydrate). For gene expression analysis, specific probe primers were designed and the size of the amplified product was analysed on 1 % agarose gel. Radioactive [α - ^{32} P]dCTP was incorporated into probe product by random primed labelling method by using NEBLOT kit (New England Biolabs, U.S.A.) according to the manufacturer's instructions. The labelled probe was purified using a PCR purification kit (Qiagen, The Netherlands) before addition to prehybridization buffer (7 % SDS and 0.2 M sodium phosphate buffer, pH 7.2) and incubated for 16 h at 65 °C.

Hac1 splicing

RNA was isolated and subjected to RT (reverse transcriptase)-PCR essentially as described before [29]. Briefly, the RNA (1 μ g) was allowed to bind to oligo-dT₃₅ (A/C/G) primer and reverse-transcribed using avian myeloblastosis virus RT for 1 h at 37 °C. The cDNA was used as a template for PCR using Hac1 forward primer (5'-AGGAAAAGGAACAGCGAAGG-3') and reverse primer (5'-TTCAAATGAATTCAAACCTGACT-3') at 95 °C for 5 min (initial denaturation) followed by 35 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s and a final extension of 72 °C for 5 min. The PCR products were resolved on a 2.5 % (w/v) agarose gel and visualized using an Alpha DigiDoc gel documentation system.

Microarray experiment and data analysis

Total RNA prepared as described above was labelled by the indirect Micromax NEN TSA labelling system (PerkinElmer Life Sciences, U.S.A.) according to the manufacturer's instructions. Briefly, cDNAs were labelled with biotin or fluorescein, hybridized on the microarray slides and detected by using strept-avidin and anti-fluorescein antibody conjugated with horseradish peroxidase, which enzymatically converts tyramide Cy3 and Cy5 into Cy3 and Cy5 respectively. The *S. cerevisiae* cDNA microarray slides were procured from The Microarray Center, Clinical Genomics Center, University Health Network (Toronto, ON, Canada). The slides were scanned using a Scanarray Lite scanner and data were acquired and analysed using GenepixPro. Data for each spot were corrected for background and the data from treated and reference samples were normalized to the total intensity. The genes that were up-regulated or down-regulated more than 2-fold in flip dye replicates were considered to be differentially expressed. Hierarchical clustering was performed using the commercially available software, Avadis (Strand Genomics).

RESULTS

Effect of homocysteine, cysteine and glutathione on yeast growth

The objective of the present study was to ascertain the role of thiols (homocysteine, cysteine and glutathione) on yeast growth and gene expression. Yeast was grown in synthetic minimal media supplemented with necessary amino acids in the presence of various concentrations (0.25–5 mM) of homocysteine for 30 h, to

Figure 1 Effect of various concentrations of exogenously added homocysteine on yeast growth

Overnight grown cultures of the yeast strain BY4742 were diluted to an A_{600} of 0.1 in synthetic minimal media and the growth of yeast was monitored (A_{600}) in the presence (0.25–5 mM) and absence of exogenously added DL-homocysteine (A) and cysteine (B) at various time points. The yeast growth in the presence of exogenously added cysteine, homocysteine and glutathione (the final concentration of all the thiols was 5 mM) is shown in (C). The results shown are the means $\pm$ S.D. ($n=3$).

see if homocysteine has any effect on yeast growth (Figure 1A). Aliquots were withdrawn at indicated time points and growth was monitored by measuring the A_{600} . Addition of exogenous homocysteine inhibited the growth of yeast in a concentration-dependent manner (Figure 1A). The inhibition was, however, transient with the treated cells showing a maximum growth inhibition of 73 % at 12 h and minimum growth inhibition of 13 % at 24 h, with growth recovery starting around 14 h. A similar

growth inhibitory effect was observed when the natural isomer, L-homocysteine, was exogenously added to yeast cells (results not shown). In the previous experiment, yeast cells were treated with homocysteine for a prolonged period of time. In order to see if homocysteine could inhibit yeast growth, even on shorter treatment, we grew yeast cells in the presence and absence of homocysteine for 2 or 4 h and then washed the cells after centrifugation to remove the exogenously added homocysteine. The washed yeast cells were then resuspended in minimal media and grown without exogenous addition of homocysteine. Even this short-term treatment of homocysteine (for 2 h) inhibited the growth of yeast up to 8 h after homocysteine was removed from the system (Supplementary Figure 1A at <http://www.BiochemJ.org/bj/396/bj3960061add.htm>). However, after 8 h, homocysteine failed to show a remarkable effect on yeast growth. When homocysteine was treated for 4 h, the effect as expected was much more pronounced, and even 12 h after the removal of homocysteine there was conspicuous inhibition in the yeast growth (Supplementary Figure 1B at <http://www.BiochemJ.org/bj/396/bj3960061add.htm>).

Exogenous addition of various concentrations (0.25–5 mM) of cysteine also resulted in inhibition of yeast growth in a concentration-dependent manner (Figure 1B). In order to test if the growth inhibitory effect of homocysteine and cysteine was due to a general thiol effect, homocysteine, cysteine or glutathione (all of 5 mM) was exogenously added to yeast culture and the growth was monitored at different time points (Figure 1C). Although homocysteine and cysteine inhibited the growth of yeast, glutathione did not have any effect, indicating that the inhibitory effect of homocysteine and cysteine was not a general thiol effect. Moreover, from Figure 1, it is clear that 5 mM cysteine has a greater inhibitory effect on yeast growth than homocysteine at the same concentration. While the inhibition in yeast cell growth after exogenous addition of homocysteine was transient, cysteine resulted in prolonged inhibition even at 36 h.

The concentration of intracellular homocysteine and cysteine was determined using HPLC after 12 h of incubation with these thiols as the maximum inhibitory effect was found at that time. The concentration of intracellular homocysteine increased from 1.5 pmol/ 10^7 cells to 33.9 pmol/ 10^7 cells after 12 h of exogenous addition of homocysteine, while the concentration of cysteine increased from 32.8 pmol/ 10^7 cells to 536.9 pmol/ 10^7 cells on exogenous addition of cysteine. Interestingly, exogenous addition of homocysteine appreciably increased the concentration of intracellular cysteine (from 32.8 pmol/ 10^7 cells to 269.3 pmol/ 10^7 cells), while exogenous addition of cysteine increased the intracellular concentration of homocysteine (from 1.5 pmol/ 10^7 cells to 10.5 pmol/ 10^7 cells). Thus the conversion of homocysteine into cysteine was much more facile (after exogenous addition of homocysteine) than the conversion of cysteine into homocysteine (after exogenous addition of cysteine). Exogenous addition of both cysteine and homocysteine resulted in an increase in the intracellular concentration of glutathione (results not shown).

It is known that unlike in human, in *S. cerevisiae*, homocysteine and cysteine are interconvertible. We thus wanted to see if cysteine and homocysteine could independently inhibit the growth of yeast. For this, we exogenously added cysteine to *str2Δ* strain (where the conversion of cysteine into cystathionine is blocked) and homocysteine to *str4Δ* strain (where the conversion of homocysteine into cystathionine – the precursor of cysteine – is blocked). We found that when homocysteine was added to *str4Δ* strain (Figure 2A) and cysteine to *str2Δ* strain (Figure 2B), drastic inhibition in yeast growth was observed in both the cases, 12 and 24 h after the exogenous addition. However, addition of

Figure 2 Effect of exogenously added cysteine and homocysteine on yeast deletion strains

Overnight grown cultures of the yeast deletion strains *str4Δ* (A) and *str2Δ* (B) were diluted to an A_{600} of 0.1 in synthetic minimal media and the growth of these strains was monitored (A_{600}) in the presence and absence of 5 mM homocysteine (A) and 5 mM cysteine (B) at indicated time points. The results shown are the means $\pm$ S.D. ($n = 3$).

homocysteine inhibited the growth of *str4Δ* strain by approx. 31 % after 12 h and approx. 38 % after 24 h, while the addition of cysteine to *str2Δ* strain inhibited its growth by 79 % after 12 h and by 76 % after 24 h. These results indicate that both homocysteine and cysteine can independently inhibit the growth of yeast.

Global analysis of changes in gene expression

From the above experiments, we have seen that both homocysteine and cysteine can exert growth inhibitory effects on yeast. We then wanted to study the gene expression profile in yeast after exogenous addition of the two thiols. For this, homocysteine or cysteine was added exogenously to yeast culture and RNA was extracted after 12 h of incubation (as mentioned in the Materials and methods section) since the growth inhibition was maximum at this time point (Figure 1A). Genes that were differentially expressed in replicate experiments included 115 down-regulated genes and 26 up-regulated genes in the case of homocysteine treatment (Supplementary Table 1A at <http://www.BiochemJ.org/bj/396/bj3960061add.htm>). The effect of cysteine was of comparable magnitude with 81 genes being down-regulated and 23 genes being up-regulated (Supplementary Table 1B at <http://www.BiochemJ.org/bj/396/bj3960061add.htm>). A summary of the functional classes of the affected genes (Table 1) clearly shows that nearly half of the up-regulated genes are involved in amino acid metabolism. Exogenous addition of both homocysteine and cysteine resulted in the alteration of genes of similar function even when they were not identical. The genes differentially expressed in both homocysteine and cysteine treatments are listed along with the functional roles of the genes affected in Table 1 (Supplementary Table 1). The up-regulated genes involved in carbohydrate metabolism are involved in steps that in fact contribute to amino acid metabolism (see the Discussion section). We have performed Northern-blot analysis for two carbohydrate metabolism genes [*FBA1* (fructose 1,6-bisphosphate aldolase) and *TDH2*] and two genes involved in the methionine cycle

Table 1 Overview of differentially expressed genes in yeast cells treated with homocysteine and cysteine

RNA was isolated from yeast cells grown in the presence and absence of homocysteine and cysteine and the global gene expression profile was studied using genome wide cDNA microarrays.

	Homocysteine	Cysteine
Down-regulation (fold change < 0.5)	115	81
Up-regulation (fold change > 2)	26	23
Functional classes of up-regulated genes		
Amino acid metabolism	7	12
Carbohydrate metabolism	2	2
Protein biosynthesis	2	2
Others	4	2
Unknown	11	5

Figure 3 Effect of exogenously added homocysteine on expression of yeast metabolic genes

Overnight grown cultures of the yeast strain BY4742 were diluted to an A_{600} of 0.1 in synthetic minimal media. The cells were grown in the presence and absence of 5 mM DL-homocysteine for 12 h and RNA was isolated from these cells. 20 μ g of RNA was loaded per lane for Northern-blot analysis. The upper panels in each blot represent gel loading of total RNA.

(Met6 and Met13) to confirm that these genes are up-regulated (Figure 3). As can be seen from the Figure, in agreement with the microarray results, in both cases the expressions of the genes were found to be up-regulated after treatment of the yeast cells with homocysteine for 12 h. The yeast gene expression profile obtained after exogenous addition of glutathione was found to be dissimilar when compared with the gene expression profile of homocysteine-treated cells (Figure 4).

It is generally believed that both cysteine and homocysteine, due to their thiol groups, readily oxidize to form cystine and homocystine (the autoxidized products of cysteine and homocysteine) respectively, with the concomitant generation of H_2O_2 and subsequently other reactive oxygen species. We thus wanted to see if exogenously added cysteine and homocysteine were oxidized to their respective dimers in the media. For this purpose, we determined the concentration of free reduced cysteine and homocysteine in the media. The concentration of free reduced homocysteine decreased from 4.76 to 4.10 mM during the span of 12 h, while the concentration of free reduced cysteine decreased from 4.62 to 3.78 mM during the same time period. These results indicate that under the experimental conditions only approx. 66 μ M homocysteine is oxidized, while a little less than 1 mM cysteine is oxidized. The transcriptional response of yeast cells to H_2O_2 treatment has been studied earlier. We used this dataset to compare the effect of homocysteine or cysteine treatment with the effect of H_2O_2 treatment [30]. The overall transcriptional response was dissimilar (Figure 4) with only 14 genes showing a similar response (11 down-regulated and three up-regulated) in both experiments. We also used the dye DCFH-DA to compare the presence of intracellular reactive oxygen species in untreated and thiol-treated cells. This dye reacts with H_2O_2 , other peroxides and peroxynitrite to form dichlorofluorescein and has been

Figure 4 Comparison of expression profiles under oxidative stress and glutathione addition with homocysteine treatment

The genes down-regulated (A) and up-regulated (B) on treatment with homocysteine include a small set of genes reported to be differentially expressed in response to treatment with H_2O_2 for 20 min [34]. The numbers indicate the number of genes in each group. (C) The expression profiles of genes that were differentially expressed after homocysteine treatment were compared with that of glutathione treatment (the present study) and genes that were differentially expressed after H_2O_2 treatment (retrieved from a previous report [34]) and analysed by hierarchical clustering. The cluster of genes with similar expression profiles are shown on the right.

widely used to detect reactive oxygen species [31]. The intensity of fluorescence of cells treated with thiols was found to be comparable with untreated cells. However, as expected, cells treated with H_2O_2 (positive control) showed considerably higher fluorescence intensity. These results suggest that the growth inhibitory effect of the thiols may not be due to the increased generation of reactive oxygen species.

Several studies provide evidence that homocysteine induces ER (endoplasmic reticulum) stress in mammalian cells [32,33]. We tested if the exogenous addition of homocysteine and cysteine induced ER stress. We found that addition of both homocysteine and cysteine but not glutathione induced the expression of *KAR2* (karyogamy 2), the yeast homologue of GRP78 (glucose-regulated protein, 78 kDa), which is a well-known marker for ER stress (Figure 5A). Furthermore, addition of homocysteine and cysteine resulted in the splicing of *HAC1* transcript (Figure 5B), indicating that ER stress may be responsible for the growth inhibition of yeast on exogenous addition of homocysteine and cysteine.

Figure 5 Effect of homocysteine on *KAR2* gene expression (A) and *HAC1* splicing (B)

Yeast cultures treated with cysteine and homocysteine as described before were used to prepare RNA. (A) Northern blotting for *KAR2* was performed as described in the Materials and methods section. (B) 1 µg of RNA was used for cDNA preparation. RT-PCR was carried out using primers on either side of the intron in *HAC1*.

DISCUSSION

Homocysteine is associated with a variety of disease conditions in humans [1]. However, the cellular effects of homocysteine and related metabolites like cysteine are not yet understood. Sulphur metabolism is also closely related to response to various physiological conditions like presence of metabolites, stress conditions like treatment with toxic compounds as well as regulatory processes like methylation of DNA and proteins. The yeast sulphur pathway has been extensively studied at the genetic, enzymatic and regulatory levels [27]. Here we have studied the effect of homocysteine and cysteine at the cellular and transcriptional levels.

Addition of homocysteine to yeast culture resulted in its sluggish growth up to approx. 10 h after which the rate of growth increased and after 24 h the difference in the growth between homocysteine-treated and untreated cells was minimal. Our results indicate that exogenous addition of homocysteine has little effect on the growth of yeast cells at 6 and 24 h. This is in agreement with the observations of Christopher et al. [34] who reported that exogenous addition of homocysteine had no significant effect on the growth of yeast at 6 and 24 h. However, homocysteine treatment inhibited the yeast growth at intermediate time points and the maximum inhibition was observed at 12 h. The inhibitory effect of homocysteine was concentration-dependent. We also demonstrate that yeast cell growth is inhibited even after transient treatment of yeast cells with homocysteine for 2 h followed by the removal of homocysteine from the medium.

Apart from homocysteine, we found that cysteine also inhibited the growth of yeast in a dose-dependent manner. In fact, the growth inhibitory effect of cysteine was much more pronounced than that of homocysteine and yeast cells failed to recover even 24 h after the exogenous addition of cysteine. Although elevated levels of homocysteine have been associated with a wide spectrum of diseases, until recently cysteine was not shown to be associated with these diseases. Recently, there are a few clinical studies showing that cysteine might play a role in cardiovascular diseases [9,10]. There have been isolated reports that cysteine has cytotoxic effects [11] although its cellular effects are not fully understood. To our knowledge, this is the first report showing that elevated concentrations of free reduced cysteine might play a deleterious role, at least in yeast. The inhibitory effects of cysteine and homocysteine are not due to a general thiol effect as exogenous

addition of glutathione did not affect the growth of yeast at any of the time points studied. We also demonstrate, using yeast deletion strains (*str2Δ* and *str4Δ*), that both homocysteine and cysteine can independently inhibit yeast growth. Addition of cysteine to *str2Δ* strain inhibited its growth much more than addition of homocysteine to *str4Δ* strain. This could be due to the fact that the intracellular concentration of the two thiols taken together was approx. 2.8-fold more when cysteine was added to *str2Δ* strain than when homocysteine was added to *str4Δ* strain (results not shown).

One of the mechanisms proposed for the deleterious effects of homocysteine is the generation of oxidative stress due to the formation of H_2O_2 during the oxidation of homocysteine [14]. However, under the experimental conditions, we did not observe any increase in the reactive oxygen species as evidenced by the comparable fluorescence intensities obtained after addition of DCFH-DA to control and thiol-treated cells. We also demonstrated that homocysteine itself does not oxidize to any appreciable extent in the culture medium, thus ruling out the possibility of generation of significant amounts of H_2O_2 in the medium. Earlier studies have demonstrated that the expressions of the antioxidant enzymes including glutathione peroxidase, catalase and superoxide dismutase were in fact up-regulated when yeast cells were treated with various concentrations of H_2O_2 [35]. In contrast, we found that when yeast was treated with cysteine and homocysteine, the genes coding for antioxidant enzymes are not up-regulated. In fact, glutathione peroxidase and catalase were down-regulated in the presence of homocysteine, while superoxide dismutase, thioredoxin and glutaredoxin were down-regulated in the presence of cysteine. These results are consistent with previous reports of down-regulation of glutathione peroxidase [32,36] and superoxide dismutase [37] on exogenous addition of homocysteine. If exogenous addition of homocysteine or cysteine would have resulted in oxidative stress, then it should have up-regulated the expression of the antioxidant enzymes as cells would try to minimize intracellular oxidative stress by scavenging the reactive oxygen species. Furthermore, the genes differentially expressed when yeast cells were treated with homocysteine were largely unaffected in an earlier study of gene expression profiling in yeast cells treated with H_2O_2 [31]. Thus it can be perceived that the growth inhibitory effect observed when yeast is treated with homocysteine or cysteine is probably not due to the generation or accumulation of reactive oxygen species like H_2O_2 .

The ability of homocysteine to induce unfolded protein response in human vascular endothelial cells has been linked to stress response and atherosclerosis [38]. GRP78, the mammalian homologue of *KAR2*, and other ER stress-related genes have been shown to be induced in umbilical-vein endothelial cells treated with homocysteine using microarray and differential display [32,33]. The splicing of the *HAC1* transcript, the yeast homologue of mammalian XBP1 (X-box binding protein 1), in the ER is necessary for the formation of a functional Hac1p, which induces *KAR2* expression. To study the state of unfolded protein response in yeast cells in the presence of thiols, we monitored *HAC1* splicing and induction of *KAR2*. We detected the short *HAC1* transcript in yeast cells treated with homocysteine and cysteine. When analysed using Northern hybridization, the *KAR2* transcript was found to be induced in yeast cells following treatment with homocysteine and cysteine. Further, glutathione treatment did not show induction of *KAR2*.

Transcriptional profiling of yeast in response to homocysteine and cysteine addition revealed that both the thiols can affect the cellular physiology through a wide variety of transcriptional responses. Several genes encoding the subunits of ATP synthase complex were found to be down-regulated. The ATP

Scheme 1 Up-regulated genes in the glycolysis and methionine metabolism pathway on exogenous addition of cysteine and homocysteine

Global gene expression of yeast on exogenous addition of homocysteine (5 mM) or cysteine (5 mM) resulted in the up-regulation of several genes. Some of the genes in the glycolysis and the methionine metabolism pathway that were up-regulated are highlighted (in grey).

synthase complex utilizes proton-motive force to generate ATP from ADP and inorganic phosphate. The structure of this enzyme complex consists of two major components, the membrane-bound F0 and soluble F1, each of which contains many subunits [39]. Exogenous addition of homocysteine resulted in the down-regulation of ATP5 and ATP4 of this complex. Both ATP4 and ATP5 are essential for ATP synthesis. However, they are not essential for viability in yeast. Deletion of either ATP4 or ATP5 leads to a slow growth phenotype. Addition of cysteine down-regulated ATP4, ATP14, ATP15 and ATP17. However, the minor reduction of ATP levels in the thiol-treated cells was not statistically significant (results not shown), implying that although the expression of certain subunits of the ATP synthase is down-regulated, this may not reduce the ATP level in the cell.

Exogenous addition of homocysteine resulted in the up-regulation of approx. 25 genes, while approx. 23 genes were up-regulated after treatment with cysteine. Expression of three genes in the glycolysis pathway, *FBA1*, *TDH2* and *PGK1* (3-phosphoglycerate kinase), was up-regulated. The genes coding for these three enzymes catalyse the conversion of fructose-1,6-bisphosphate to 3-phosphoglycerate in three steps (Scheme 1). 3-Phosphoglycerate can either be converted into 2-phosphoglycerate in the glycolysis pathway or it can be converted into

3-phospho-hydroxy pyruvate, which is the first step in the serine biosynthesis pathway. Interestingly, *GPM1* (glycerate phosphomutase 1) that catalyses the conversion of 3-phosphoglycerate into 2-phosphoglycerate in the glycolytic pathway is down-regulated, while the gene *SER3* (3-phosphoglycerate dehydrogenase) that is responsible for the conversion of 3-phosphoglycerate into 3-phospho-hydroxy pyruvate in the serine biosynthesis branch is up-regulated when cells were treated with homocysteine. Furthermore, *SER1* that converts 3-phospho-hydroxy pyruvate into 3-phosphoserine is up-regulated. Also up-regulated in the case of cysteine and homocysteine is the gene *SHM2* (serine hydroxymethyltransferase 2) that converts tetrahydrofolate into 5,10-methylene tetrahydrofolate in the folate cycle. This is a one-carbon transfer reaction where serine is converted into glycine (Scheme 1). The serine biosynthesis pathway links the glycolysis pathway with the folate cycle. The genes *MET13* (methylene-tetrahydrofolate reductase 13) that converts 5,10-methylene tetrahydrofolate into 5-methyl tetrahydrofolate and *MET6* that catalyses the remethylation of homocysteine to methionine are also up-regulated. This is consistent with an earlier report where Kacprzak et al. [40] demonstrated that increased intracellular concentration of homocysteine resulted in higher expression of *MET6* gene.

Cbf1p, Met28p, Met31p and Met32p are transcriptional activators required for the regulation of the MET genes in yeast. Cbf1p binds to DNA through a CACGTG motif in promoter proximal regions of MET genes and appears to participate in both promoter recruitment and chromatin modification [41]. We searched for the CACGTG motif in the other genes up-regulated in our studies along with the MET genes. We find CACGTG motif within 500 bp upstream of *GLK1* (glucokinase 1), *TDH2* and *PGK1*, which are involved in carbohydrate metabolism. These elements may thus be involved in the co-regulation of these genes with the MET genes.

From the gene expression data, it is apparent that exogenous addition of cysteine and homocysteine up-regulated the glycolysis pathway genes since glycolysis is the source of serine. One of the reasons for the increased demand for serine is the need to increase the metabolism of homocysteine. Serine as mentioned above is necessary for the formation of 5,10-methylene tetrahydrofolate. Furthermore, serine promotes catabolism of homocysteine via condensation reaction to form cystathionine in the first step of the transsulphuration pathway. Thus serine is needed for both remethylation and transsulphuration branches of homocysteine metabolism. Interestingly, the intracellular concentration of homocysteine was found to be lower at 24 h than at 12 h after exogenous addition of homocysteine (results not shown), which indicates that cells try to reduce the intracellular concentration of thiols by up-regulating the genes involved in their metabolism.

Thus, by monitoring yeast growth after exogenous addition of cysteine and homocysteine and from the whole genome expression analysis coupled with measurement of intracellular thiols, we have shown that homocysteine and cysteine both inhibit the growth of yeast and this involves specific changes in gene expression in yeast. The growth inhibitory effect of cysteine or homocysteine is not due to oxidative stress produced by the generation and/or accumulation of H_2O_2 . However, it appears that ER stress might be involved in the homocysteine- and cysteine-mediated growth inhibition in yeast. Furthermore, we also conclude that cells, as part of an adaptive mechanism, try to reduce these toxic thiols by up-regulating the genes that are responsible for their metabolism.

We thank Dr Dwaipayan Bharadwaj, Dr Souvik Maiti and Jitender Kumar (Institute of Genomics and Integrative Biology, New Delhi, India) and Dr Sushil Kumar (University of Jammu) for a critical review of this paper. This work was supported by grants from CSIR (Council of Scientific and Industrial Research), India (CMM 0018 to S.S. and B.P.). A.K. was supported by a fellowship from CSIR.

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Received 26 August 2005/19 January 2006; accepted 24 January 2006

Published as BJ Immediate Publication 24 January 2006, doi:10.1042/BJ20051411