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Effect of nonprotein thiols on protein synthesis in isolated rat hepatocytes

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Abstract. The ability of nonprotein thiols to modulate rates of protein synthesis was investigated in isolated rat hepatocytes. Addition of cysteine stimulates protein labelling by [^{14}C]Leucine. Glutathione depletion, induced by in vivo administration of L-buthionine sulfoximine and diethylmaleate, did not alter the effect of cysteine, although it decreased the rate of protein synthesis by 32%. The effect of cysteine on protein synthesis does not seem to be related to a perturbation of the redox state of the NAD^+/NADH system or to changes in the rate of gluconeogenic pathway. The following observations indicate that cysteine may stimulate protein synthesis by increasing intracellular levels of aspartate: 1. Amino-oxyacetate, an inhibitor of pyridoxal-dependent enzymes, inhibits protein labelling and decreases aspartate cellular content, whereas most amino acids accumulate or remain unchanged; 2. Cysteine, in the absence or in the presence of amino-oxyacetate, stimulates protein labelling and induces aspartate accumulation, although most amino acids diminish or remain unchanged.
Key words. Cysteine; glutathione; protein synthesis; aspartate; thiols.

Abbreviations. GSH, reduced glutathione; GSSG, oxidized glutathione; GSH ester, glutathione monoethyl ester; BSO, L-buthionine sulfoximine; DEM, diethylmaleate; NAC. N-acetyl cysteine; AOA, amino-oxyacetate.

Nonprotein thiols are required for efficient protein synthesis in many cells and cell-free systems[1]. Glutathione (GSH), the most prevalent nonprotein thiol in eucaryotic cells, is essential for many cellular functions, protecting thiol groups in enzymes and other proteins[1,2]. Indeed, protein synthesis is inhibited when GSH is oxidized[3,4]. Moreover, synthesis of polypeptides in ribosomal preparations of brain or liver is inhibited by glutathione disulfide (GSSG)[5]. Thus, it was postulated that a depletion of GSH and/or an increase in intracellular levels of GSSG lead to the inhibition of protein synthesis[6]. Furthermore, GSH has been postulated as a physiological reservoir of cysteine[7] and as a source of amino acids for protein synthesis[8]. Interestingly, Szent-Györgyi et al.[9] used methylglyoxal derivatives to block intracellular thiol groups and observed that the inhibition in protein synthesis they found could be reversed by addition of cysteine. Therefore, thiols appear to be involved in regulating protein synthesis. However, although most individual amino acids are able to stimulate protein synthesis in rat hepatocytes[10], the potential role of the different physiological thiols is unclear. In this paper, we report the effects of nonprotein thiols on protein synthesis. In addition, possible correlations between gluconeogenesis, intracellular amino acid content and protein synthesis were also investigated.

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Materials and methods

Chemicals. [^{14}C]Leucine (325 Ci/mol) was purchased from the Radiochemical Centre (Amersham, Bucks, UK). Cycloheximide, amino oxyacetate (AOA), L-buthionine sulfoximine (BSO) and diethylmaleate (DEM) were from Sigma Chemical Co. (St. Louis, MO, USA). Glutathione monoethyl ester (GSH ester) was synthesized as described by Anderson and Meister[11]. Standard analytical-grade chemicals were obtained from Merck (Darmstadt, Germany). Enzymes and coenzymes were from Boehringer (Mannheim, Germany). Silicone oils, AR 200 and SF96: 100 were from Serva (Heidelberg, Germany) and from Klöckner (Duisburg, Germany), respectively.

Isolation and incubation of hepatocytes. Hepatocytes were isolated from 48 h-starved Wistar rats (220–250 g), according to Berry and Friend[12], and suspended (about 10–12 mg dry wt./ml) at 37 °C in Krebs-Henseleit bicarbonate medium (pH 7.4) containing 1.3 mM $CaCl_2$. The gas atmosphere was 95% O_2–5% CO_2. After incubation, the cells were either separated from the medium by the fractionation technique described by Zuurendonk and Tager[13] (for determination of intracellular and extracellular radioactivity, and/or metabolites), or the whole sample (cells + medium) was treated with perchloric acid (2% final concentration) directly for determination of total metabolite levels or protein labelling. In all our experimental conditions we determined cell membrane integrity by measuring Try-

Table 1. Effect of nonprotein thiols on protein synthesis in isolated rat hepatocytes.

Additions	Protein synthesis (d.p.m./h per mg prot.)	% of control
None	299 ± 14 (5)	100
Cysteine 1 mM	585 ± 40 (5)**	196
Cystine 1 mM	311 ± 27 (5)	104
GSH 1 mM	343 ± 21 (5)**	115
GSH ester 1 mM	439 ± 35 (5)**	147
Cysteamine 1 mM	323 ± 16 (4)	108
N-acetylcysteine 1 mM	546 ± 34 (5)**	183
2-Mercaptoethanol 1 mM	452 ± 33 (4)**	151
1,4-Dithiothreitol 1 mM	397 ± 18 (4)**	133

Hepatocytes were incubated for 60 minutes in the presence of 5 mM leucine. Results are means ± SD for the number of experiments in parentheses. Statistical significance was determined by Student's t-test. **$p < 0.01$ comparing each condition against the control.

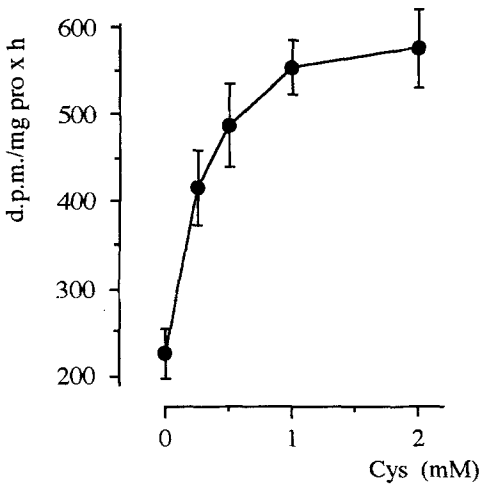

Figure 1. Effect of different concentrations of cysteine on protein synthesis in isolated rat hepatocytes. Hepatocytes were incubated in the presence of 5 mM leucine and protein synthesis was measured as described in Methods. The rate of protein labelling was calculated for the 30–60 minutes interval. The incubation time was 60 minutes. Values are means ± SD of six different hepatocyte preparations.

pan Blue exclusion (cell viability was always greater than 93%) and leakage of lactate dehydrogenase (always less than 4% of the total activity measured in the incubation flask) from incubated cells.

Protein synthesis. It was measured as the incorporation of [^{14}C]Leucine into the protein acid-insoluble fraction under conditions of constant specific radioactivity (5 mM and 25 μCi/l). The rate of protein synthesis was expressed as d.p.m./h per mg protein.

Induction of GSH depletion. GSH depletion was achieved by injecting rats i.p., 1 hour prior to the isolation of hepatocytes, with a mixture of BSO and DEM (5 mM and 1 mM, respectively, were the final concentrations in the water space of the animals) diluted in physiological saline. To avoid restoration of GSH levels in cells isolated from treated rats, we included BSO (5 mM) in the incubation medium.

Assay of metabolites. GSH and GSSG were measured as described by Asensi et al.[14]. Total cellular proteins were measured by the method of Lowry et al.[15]. Glucose, lactate pyruvate and ATP were determined by standard enzymatic methods. Amino acids were measured with a Waters H.P.L.C. system. Lactate dehydrogenase activity was measured in cell-free supernatants as previously described[16].

Expression of results and statistical significance. The results are expressed as means ± SD for the indicated number of different preparations. The statistical significance of differences was assessed by Student's t-test.

Results and discussion

Effect of nonprotein thiols on protein synthesis

As shown in table 1, cysteine, N-acetyl cysteine (NAC), GSH and GSH ester stimulated protein labelling significantly in isolated rat hepatocytes (all these effects are abolished by addition of 1 mM cycloheximide to the incubation medium – results not shown). On the other hand, neither cystine nor cysteamine showed any significant effects (table 1). The highest percentage of protein synthesis stimulation was obtained in the presence of cysteine, which caused an increase in the rate of protein labelling of 100% over the controls (table 1). The effect elicited by cysteine is concentration dependent and was already significant at 0.25 mM (fig. 1). This fact is interesting since we previously found that the intracellular concentration of free cysteine in hepatocytes is very low (0.1–0.4 mM, ref. 17). GSH cannot cross the plasma membrane, but it can be degraded by the gamma-glutamyl transpeptidase present on the cell surface and yield free cysteine, which may enter the cell (see for instance ref. 2). Nevertheless, GSH appears less effective than GSH ester or NAC, which cross the cell membrane easily and give rise to cysteine after rapid metabolization. The low stimulation of protein synthesis elicited by GSH can easily be explained by the fact that hepatocytes show low gamma-glutamyl transpeptidase activity[18].

Being itself an amino acid, is the effect of cysteine on protein synthesis due to increased substrate availability? To answer this question, we tested the effect of non-physiological nonprotein thiols, such as 2-mercaptoethanol or 1,4-dithiothreitol (table 1). We found that both compounds stimulate protein synthesis significantly. This proves that protective agents for -SH groups, by their antioxidant properties or by modulating the intracellular concentration of some key amino acid(s), can influence the rate of protein synthesis.

Table 2. Effect of cysteine and glutathione depletion on protein synthesis in isolated rat hepatocytes.

	Protein synthesis (d.p.m./mg prot. × h)	
	− Cys	+ 1 mM Cys
Control	299 ± 14	612 ± 26**
Glutathione depletion	203 ± 12^{++}	392 ± 38**$^{++}$

Results are expressed as means ± SD for five different experiments. Statistical significance was determined by Student's t-test: **$p < 0.01$ comparing the effect of cysteine in both conditions (control and glutathione depletion); $^{++}p < 0.01$ comparing the effect of glutathione depletion in both conditions (presence and absence of 1 mM cysteine).

Table 3. Effect of cysteine and amino-oxyacetate (AOA) on protein synthesis in isolated rat hepatocytes in the presence of 10 mM lactate and 1 mM pyruvate.

	Protein synthesis (d.p.m./mg prot. × h)	
	− Cys	+ 1 mM Cys
− AOA	538 ± 23	612 ± 26**
+ 0.5 mM AOA	147 ± 17^{++}	367 ± 41**$^{++}$

Hepatocytes were incubated in presence of 10 mM lactate and 1 mM pyruvate. Results are expressed as means ± SD for five different experiments. Statistical significance was determined by Student's t-test: **$p < 0.01$ comparing the effect of cysteine in both conditions (presence and absence of 0.5 mM AOA); $^{++}p < 0.01$ comparing the effect of glutathione depletion in both conditions (presence and absence of 1 mM Cys).

Protein synthesis in GSH-depleted cells

As stated above, the initiation and elongation of proeins is inhibited when GSH is oxidized[3]. This inhibition may be attributable to an increase in GSSG concentration, since when GSSG is reduced, elongation resumes[6]. However, GSH is present in cells at concentrations that are much higher than GSSG[2,14,19]. Thus, under physiological conditions, the low nanomolar concentration of GSSG within the cells does not affect protein synthesis. But does GSH play a role in protein synthesis? To address this question we induced a depletion in the cellular content of GSH by using a mixture of BSO, a specific inhibitor of gamma-glutamylcysteine synthetase[20], and DEM, a substrate for the GSH transferase[21]. By this procedure (see Methods), we reached a GSH concentration of 0.9 ± 0.2 µmol/g ($n = 10$) in the isolated rat hepatocytes (approximately 16% of the GSH content in hepatocytes from fed rats). We also measured the GSSG concentration, which was 17.8 ± 1.4 nmol/g (approximately 50% lower than that found in the absence of BSO and DEM). Neither GSH nor GSSG varied significantly during the incubation time (60 minutes; $p > 0.05$). Moreover, under conditions of glutathione depletion, addition of cysteine (1 mM) did not alter the values of GSH and GSSG mentioned above. GSH depletion decreased protein labelling by approximately 32–33%, either in absence or in the presence of 1 mM cysteine (table 2). These results suggest that GSH, by itself or as a source of cysteine[8], plays a role in regulating protein synthesis.

Interrelationship between gluconeogenesis, cellular amino acid content and protein synthesis

According to Meijer et al.[22], AOA may lead a more reduced cytosolic $NAD^+/NADH$ system, and to an inhibition of gluconeogenesis from reduced precursors

Table 4. Effect of cysteine and amino-oxyacetate on the intracellular concentration of amino acids.

Amino Acid	Control		+ AOA 0.5 mM	
	− Cys	+ Cys 1 mM	− Cys	+ Cys 1 mM
Aspartate	0.61 ± 0.05	0.78 ± 0.14^{+}	0.44 ± 0.05**	2.86 ± 0.08^{++}
Glutamate	3.19 ± 0.45	2.34 ± 0.39^{+}	4.91 ± 0.56**	2.86 ± 0.33^{++}*
Serine	0.51 ± 0.04	0.25 ± 0.02^{++}	1.56 ± 0.10**	1.04 ± 0.08^{++}**
Glutamine	0.66 ± 0.11	0.45 ± 0.07^{+}	0.70 ± 0.07	0.44 ± 0.05^{++}
Glycine	0.93 ± 0.13	0.41 ± 0.06^{++}	2.37 ± 0.40**	1.36 ± 0.12^{++}**
Threonine	0.14 ± 0.01	0.06 ± 0.02^{++}	0.37 ± 0.05**	0.18 ± 0.01^{++}**
Citrulline	1.46 ± 0.09	0.98 ± 0.12^{++}	2.00 ± 0.37*	0.93 ± 0.10^{++}
Alanine	0.71 ± 0.08	0.87 ± 0.05^{++}	0.74 ± 0.09	0.76 ± 0.08*
Taurine	0.91 ± 0.12	1.47 ± 0.14^{++}	1.08 ± 0.10*	1.64 ± 0.17^{++}
Tyrosine	1.02 ± 0.09	0.96 ± 0.08	1.07 ± 0.06	0.97 ± 0.11
Phenylalanine	0.59 ± 0.10	0.52 ± 0.07	0.58 ± 0.11	0.56 ± 0.07
Isoleucine	1.00 ± 0.07	0.89 ± 0.15	0.92 ± 0.09	0.92 ± 0.13
Leucine	0.35 ± 0.04	0.36 ± 0.03	0.30 ± 0.06	0.30 ± 0.03
Ornithine	0.55 ± 0.05	0.62 ± 0.07	0.66 ± 0.06*	0.80 ± 0.11^{+}*
Lysine	0.24 ± 0.03	0.38 ± 0.04^{++}	0.41 ± 0.05**	0.80 ± 0.09^{++}**

Liver cells were incubated for 60 minutes in the presence of 5 mM leucine. The results are means ± SD for five different experiments. For calculations, cytosolic and mitochondrial water contents of 2.0 and 0.2 ml/g dry wt. were assumed[26]. Intracellular concentration of amino acids at time 0 was not significantly different from control values obtained in the absence of cysteine (results not shown). Statistical significance was determined by Student's t-test: *$p < 0.05$, **$p < 0.01$ comparing both conditions where cysteine was not present, and both conditions where cysteine was present; $^{+}p < 0.05$, $^{++}p < 0.01$ comparing both conditions under control and both conditions where amino-oxyacetate was present.

such as lactate. Under our experimental conditions, in the presence of lactate (10 mM) and pyruvate (1 mM), the aminotransferase inhibitor (0.5 mM) promoted a rise in the (lactate)/(pyruvate) ratio from 10.7 ± 0.02 ($n = 5$) to 51.5 ± 2.3 ($n = 5$; $p < 0.01$). When added, cysteine (1 mM) did not alter this value, but induced a significant increase from 51.5 ± 2.3 ($n = 5$) to 66.9 ± 2.6 ($n = 5$; $p < 0.01$) in the (lactate)/(pyruvate) ratio found in the presence of AOA. Changes in the (lactate)/(pyruvate) ratio were mainly due to variations in pyruvate availability. The rate of glucose production fell from 1.02 ± 0.03 ($n = 6$) to 0.11 ± 0.04 ($n = 6$; $p < 0.01$) in the presence of AOA. These rates of glucose production were not significantly altered by addition of cysteine. Moreover, cysteine did not induce changes in the rate of cellular oxygen uptake or ATP contents (results not shown). Thus, the effect of cysteine on protein labelling is not related either to a perturbation of the redox state of the NAD^+/NADH system nor to changes in the rate of the gluconeogenic pathway.

Intracellular aspartate availability has been postulated as a rate-limiting step for protein synthesis in isolated rat hepatocytes[10]. We tested whether the effect of cysteine on protein synthesis could be related to changes in the intracellular concentration of free aspartate or some other key amino acids. For this purpose, we used amino-oxyacetate (carboximethoxylamine; AOA), a well-characterized inhibitor of pyridoxal-dependent enzymes[23] and an inhibitor of protein synthesis in liver[24,25]. In the presence of lactate (10 mM) and pyruvate (1 mM), AOA (0.5 mM) decreased protein labelling to approximately 27% of its control rate (table 3). Under these conditions, cysteine (1 mM) stimulated protein labelling (table 3). We also found that in the presence of AOA, most amino acids accumulate or remain unchanged, aspartate being the only exception (table 4). When cysteine was added to the incubation medium, most amino acids were diminished or remained unchanged, but aspartate accumulated (table 4; similar results were obtained by incubating hepatocytes in the presence of 2-mercaptoethanol – results not shown). Moreover, this effect of cysteine was found both in the presence and in the absence of AOA (table 4). These results support the idea of a control role of aspartate on protein synthesis, and suggest an involvement of cysteine in the regulation of the transamination reaction leading to aspartate formation. We conclude that the effect of cysteine on protein synthesis may be due, at least in part, to an increase in aspartate availability within the cell.

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1 Ochoa, S., Archs Biochem. Biophys. *223* (1983) 325.
2 Meister, A., Science *220* (1983) 470.
3 Zehavi-Willner, T., Israel J. med. Sci. *9* (1973) 129.
4 Kan, B., London, I. M., and Levin, D. H., J. biol. Chem. *263* (1988) 15652.
5 Murthy, M. R. V., Biochim. biophys. Acta *119* (1966) 586.
6 Kosower, N. S., and Kosower, E. M., Int. Rev. Cytol. *58* (1978) 109.
7 Tateishi, N., Higashi, T., Naruse, A., Nakashima, K., Shiozaki, H., and Sakamoto, Y., J. Nutr. *107* (1977) 51.
8 Pocius, P. A., Clark, J. H., and Baumrucker, C. R., J. Dairy Sci. *64* (1981) 1551.
9 Szent-Györgyi, A., Együd, L. G., and McLaughlin, J. A., Science *155* (1967) 539.
10 Pérez-Sala, D., Bengoa, B., Martín-Requero, A., and Parrilla, R., Biochem. J. *242* (1987) 485.
11 Anderson, A., and Meister, A., Analyt. Biochem. *183* (1989) 16.
12 Berry, N. M., and Friend, D. S., J. Cell Biol. *43* (1969) 506.
13 Zuurendonk, P. F., and Tager, J. M., Biochim. biophys. Acta *333* (1974) 393.
14 Asensi, M., Sastre, J., Pallardó, F. V, García de la Asunción, J., Estrela, J. M., and Viña, J., Analyt. Biochem. *217* (1994) 323.
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
16 Bergmeyer, H. U., and Bernt, E., in: Methods of Enzymatic Analysis, p. 574. Ed. H. U. Bermeyer., Verlag Chemie, Weinheim, and Academic Press Inc., New York and London 1974.
17 Estrela, J. M., Gil, F., Vila, J. M., and Viña, J., Am. J. Physiol. *255* (1989) E801.
18 Viña, J. R., Palacín, M., Puertes, I. R., Hernández, R., and Viña, J., Am. J. Physiol. *257* (1989) E916.
19 Deneke, S. M., and Fanburg, B. L., Am. J. Physiol. *257* (1989) L163.
20 Griffiths, P. A., and Lloyd, J. B., Biochem. biophys. Res. Commun. *89* (1979) 428.
21 Boyland, E. L., and Chasseaud, L. F., Biochem. J. *104* (1967) 95.
22 Meijer, A. J., Gimpel, J. A., Deleeuw, G., Tischler, M. E., Tager, J. M., and Williamson, J. R., J. biol. Chem. *253* (1978) 2308.
23 Rognstad, R., and Katz, J., Biochem. J. *116* (1970) 483.
24 Seglen, P. O., and Solheim, A. E., Biochim. Biophys. Acta *520* (1978) 630.
25 Poli, A., Gordon, P. B., Schwarze, P. E., Grinde, B., and Seglen, P. O., J. Cell Sci. *48* (1981) 1.
26 Tischler, M. E., Hecht, P., and Williamson, J. R., Archs Biochem. Biophys. *181* (1977) 278.