GLUTATHIONURIA: INBORN ERROR OF METABOLISM DUE TO TISSUE DEFICIENCY OF GAMMA-GLUTAMYL TRANSPEPTIDASE

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Received May 7,1975

Summary

A patient has been discovered with glutathionemia and marked glutathionuria. Serum concentrations of individual aminoacids were normal and percent renal resorption of these amino acids fell within or very close to the normal range under ordinary dietary conditions. Cultured skin fibroblasts from the patient manifested an extreme reduction in the activity of gamma-glutamyl transpeptidase measured with glycylglycine, cystine, or methionine acceptors and the artificial substrate gamma-glutamyl-nitroanilide, and in an assay using ^{35}S-glutathione as substrate. A "new" human enzyme deficiency state has thus been characterized. Further studies of this disorder should permit clarification of the possible role of this transpeptidase in human amino acid transport and in glutathione metabolism.

Introduction

Glutathione (L-γ-glutamyl-L-cysteinylglycine) is present in virtually all mammalian cells. The enzyme gamma-glutamyl transpeptidase, the presence of which in certain human and other mammalian tissues has been recognized for some time (1,2,3), acts as a glutathionase and catalyzes the transfer of the glutamyl moiety of glutathione to a variety of acceptor molecules including water, certain L-amino acids, and peptides (4,5). Several studies have presented data compatible with the important but unproven hypothesis that this enzyme, in conjunction with the gamma-glutamyl (or glutathione) cycle, plays a crucial role in cellular amino acid transport (5,6,7,8).

This report describes an extreme deficiency of gamma-glutamyl transpeptidase in fibroblasts from a human subject with glutathionemia and glutathionuria in whom neither significant aminoaciduria nor aminoacidemia was demonstrated.

Materials and Methods

The patient is a mildly retarded adult male who came to the attention of one of us (S.I.G.) during routine screening of institutionalized individuals for disorders of amino acid metabolism. An abnormal ninhydrin-positive metabolite noted in urine was identified as glutathione and quantitated in serum and urine as previously described (9). Amino acids in serum and urine were measured using a Beckman model 120 automatic amino acid analyzer; serum samples were promptly deproteinized with sulfosalicylic acid (65 mg/ml) and all samples were stored at -20° C until analyzed.

Cultured fibroblasts were obtained from skin biopsies of the patient and multiple controls. The cells were propagated by standard methods in either Eagle's Minimal Essential Medium with added non-essential amino acids or in Medium F 10, and were assayed for gamma-glutamyl transpeptidase activity several days after cell passage and before confluency had been attained. Two assay techniques were employed, as detailed in the table legends; one method utilized the synthetic substrate gamma-glutamyl-p-nitroanilide (Sigma) (10) and the other, developed for this investigation, measured formation of ^{35}S-cyst[e]inylglycine and ^{35}S-cyst[e]ine from ^{35}S-glutathione (obtained from Amersham and appropriately tested for purity by high voltage electrophoresis).

Results

Serum and urine concentrations and calculated percent tubular resorption of individual amino acids during normal dietary intake are given in table 1. Serum amino acid concentrations of the glutathionuric patient were all normal except for a modest reduction in the sum of asparagine and glutamine and a roughly comparable increase in glutamic acid, presumably reflecting hydrolysis of glutamine to glutamate during sample storage and preparation. Percent renal tubular resorption was normal for 14 amino acids and fell only slightly below the normal range for 4 amino

Table 1. Serum and urine amino acid concentrations and renal tubular resorption of amino acids in the patient and in normal adults.

Amino Acid	Serum Concentration (umoles/ml)		Urine Conc. (umoles/ml)	% Tubular Resorption	
	Patient	Normal*	Patient	Patient	Normal*
OH-proline	.010	0-.010	N.D.**	99+	99+
Aspartate	.016	0-.024	<.010	>97.8	85-98
Threonine	.137	.079-.246	.076	98.0	97-99
Serine	.101	.067-.193	.140	95.1	97-99
$AspNH_2$+$GluNH_2$	.174	.413-.690	.310	93.7	99+
Proline	.191	.100-.442	N.D.	99+	99+
Glutamate	.429	.014-.200	<.01	>99.9	99+
Glycine	.275	.120-.553	.312	96.0	94-99
Alanine	.384	.209-.659	.242	97.8	99+
Valine	.206	.050-.315	<.01	>99.8	99+
1/2 Cystine	N.D.	0-.141	N.D.	----	99+
Methionine	.023	.003-.040	<.01	>98.5	98-99+
Isoleucine	.057	.035-.097	<.01	>99.4	99+
Leucine	.115	.071-.175	<.01	>99.7	99+
Tyrosine	.054	.021-.087	.052	96.7	98-99+
Phenylalanine	.055	.037-.115	.034	97.8	99+
Ornithine	.053	.029-.125	<.01	>99.3	99+
Lysine	.143	.082-.236	.011	99.7	99+
Histidine	.100	.031-.106	.030	98.9	92-98
Arginine	.081	.021-.137	<.01	>99.6	99+

*Range based on values from our laboratories and ref. 18.

**Not detected.

acids and for the sum of asparagine and glutamine. No major deviation from normal was observed in the serum concentrations or renal clearances of any quantitated amino acids.

The concentration of glutathione (reduced + oxidized) in serum from the patient was 1.4-2.4 micrograms/ml (normal 0.33-0.53) and in urine was 86-125 micrograms/ml (normal 0.03 - 0.10). The molar ratio of the oxidized to the reduced form in serum was 1.5:1 and in urine was 1.3:1. During a one-hour period in which endogenous creatinine clearance was 90 ml/min/1.73 m^2, the clearance of reduced and oxidized glutathione were 210 and 192 ml/min/1.73 m^2 respectively. The total urinary excretion of glutathione during this one hour interval was 15 mg.

On another occasion also during the ingestion of a normal institutional diet, the excretion of glutathione during a 24 hour urine collection was 850 mg. These data suggest that in this disorder glutathionuria is secondary to glutathionemia, and that glutathione is secreted by or leaks from renal cells into the glomerular filtrate.

Gamma-glutamyl transpeptidase activity was measured in washed, freeze-thawed cultured fibroblasts from the patient and seven controls using the artificial substrate gamma-glutamyl-p-nitroanilide (10) (table 2). The reaction is linear and

Table 2. Gamma-glutamyl transpeptidase activity in fibroblast lysates from patient and 7 controls using gamma-glutamyl-p-nitroanilide substrate (expressed as nanomoles p-nitroaniline released/mg protein/hour due to the addition of acceptor).

	Cell line	Acceptor		
		20mM glycylglycine	20mM cystine	20mM methionine
Controls	A	140	92	51
	B	189	100	60
	C	75	58	32
	D	148	78	38
	E	63	54	26
	F	170	116	63
	G	116	72	40
Control mean		129	81	44
Glutathionuric		Undetectable (<5)	Undetectable	Undetectable

Incubations were at 37 degrees C. for 4 hrs., but in separate experiments no activity was found in glutathionuric cells even after 24 hours incubation. Assay mixture concentrations in a final volume of 0.5 ml: 100 mM Tris pH 9.0; 10mM $MgCl_2$; 2mM or 20mM acceptor; 1mM gamma-glutamyl-p-nitroanilide; and 0.3 - 0.5 mg cell protein. The reaction was stopped with 50µl 50% trichloracetic acid and after 5 minutes 450µl 2N acetic acid was added. The optical density was read at 410mµ after centrifugation. Protein was assayed by the method of Lowry (19).

proportional to cell protein up to at least 6 hours, with modest deviation from linearity at 24 hrs. As found in other studies, glycylglycine and L-methionine were good acceptors of the glutamyl group, markedly increasing the rate of release of p-nitroaniline from the colorless substrate. We have recently demonstrated that in a variety of human tissues cystine is also a potent activator of this reaction (11,12,13), but because of relative insolubility it was included at 2mM instead of 20 mM final concentration in the assay. There was no detectable gamma-glutamyl transpeptidase activity in fibroblasts from the glutathionuric patient with any of the above acceptors nor in the absence of added acceptor. A lower limit of 5% of the control mean activity would have been detectable in the mutant cells in the presence of glycylglycine, had such activity been present.

To prove unequivocally that gamma-glutamyl transpeptidase activity was deficient when assayed with its natural substrate glutathione, we developed a sensitive radio-chemical assay using ^{35}S-glutathione (table 3), with glycylglycine as glutamyl acceptor. Cold cysteine was added to trap any cyst[e]ine which could have resulted from the action of cysteinylglycinase on ^{35}S-cysteinylglycine produced by the primary reaction, but in fact almost all end product label was found as cysteinylglycine. The patient's fibroblasts were compared to 6 control cell lines, and no activity was detectable in his cells (<4% of control mean activity).

Discussion

Glutathionuria must be rare, being thoroughly documented only in the present case, although an abstract has appeared describing a possibly similar patient in Ireland (14). Lack of currently available details of family history precludes conclusions as to the heritability of this inborn defect. Our patient represents an unusually instructive experiment of nature in view of current limited knowledge about the metabolism of glutathione and the possible role of gamma-glutamyl transpeptidase in amino acid transport mediation (5,6). The investigations of

Table 3. Gamma-glutamyl transpeptidase activity in fibroblast lysates from patient and 6 controls using ^{35}S-glutathione substrate and glycylglycine acceptor (expressed as nanomoles cyst[e]inylglycine + cyst[e]ine formed/mg protein/hour).

	Cell line	Activity
Controls	I	36
	II	46
	III	46
	IV	85
	V	26
	VI	84
Control mean		54
Glutathionuric		Undetectable (<2)

Data for each line are the results of duplicate assays. Incubation was under nitrogen for 6 hours at 37 degrees C. Assay mixture concentrations in a final volume of 0.5ml: 100 mM Tris pH 8.0; 1mM EDTA; 5mM ^{35}S-GSH (reduced glutathione); 20mM glycylglycine; 1mM cysteine; 1-2 mg fibroblast protein. GSH remained almost fully reduced during the incubation period. After incubation, 50µl aliquots were removed and mixed with 50µl of 20mM dithiothreitol in 20mM phosphate pH 7.0. After 2 minutes, 200µl of 20mM N-ethylmaleimide in the same buffer was added. After 2 more minutes, the mixture was acidified with 200µl 7.8% formic acid. 40µl aliquots of the final mixtures were subjected to high voltage paper electrophoresis (3500 V x 2 hours). Counts in the GSH-NEM, cysteinyl-NEM, and cysteinylglycine-NEM areas were quantitated by liquid scintillation counting. Percent increase over 0 time in the two reaction products was calculated and activity units computed. Protein was assayed by the method of Lowry (19).

tissue levels reported here demonstrate that an extreme deficiency of gamma-glutamyl transpeptidase, as measured with synthetic and natural substrate, constitutes the primary enzymatic basis for this disorder. These data are compatible with the previously reported indications of reduced levels of gamma-glutamyl transpeptidase in this patient's serum measured with synthetic substrate only (15) and with his prominent glutathionemia and glutathionuria. If, as seems very likely, the enzyme deficiency in this patient is generalized and includes the

kidney, the absence of gross abnormalities of renal amino acid clearance at normal serum amino acid concentrations could be interpreted as suggesting that gamma-glutamyl transpeptidase may not be an important mediator of amino acid transport in man. It remains possible however that extremely small amounts of transpeptidase activity, below levels of detection by the methodology reported here, could permit sufficient functioning of the gamma-glutamyl cycle that abnormalities of amino acid metabolism would not be evident by analysis of blood and urine samples obtained only under normal conditions. Detailed study of amino acid clearance rates by the kidney in this patient during amino acid loading *in vivo* and of the kinetics of amino acid transport by his cultured fibroblasts *in vitro*, and additional studies in progress at the present time, should permit critical evaluation of the possible role (16,17) of this transpeptidase in amino acid transport in man and add substantially to our knowledge about human glutathione metabolism.

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