Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 504-511

TREATMENT OF GLUTATHIONE SYNTHETASE DEFICIENT FIBROBLASTS BY INHIBITING γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY WITH SERINE AND BORATE

Stephen P. Spielberg, Jean DeB. Butler,* Kay MacDermot, and Joseph D. Schulman

Departments of Pharmacology and Experimental Therapeutics, and Pediatrics, Johns Hopkins School of Medicine, Baltimore, Maryland 21205;

and

Section on Human Biochemical and Developmental Genetics, NICHD, NIH, Bethesda, Maryland 20014

Received June 14, 1979

SUMMARY

Glutathione synthetase deficiency results in decreased cellular glutathione content and consequent overproduction of 5-oxoproline. L-serine in borate buffer inhibits γ-glutamyl transpeptidase, the major catabolic enzyme for glutathione. Treatment of glutathione synthetase deficient fibroblasts with 40mM serine and borate for 24 hours produced more than a 2-fold increase in cellular glutathione content. L-serine alone led to a smaller increase in glutathione level, and borate alone was without effect. On exposure to serine and borate, 5-oxoproline formation from L-glutamate was decreased to normal levels in glutathione synthetase deficient fibroblasts, presumably secondary to feedback inhibition of γ-glutamylcysteine synthetase by the increased intracellular glutathione concentration. Cellular free amino acid content was generally unaffected by such exposure although increases were observed in serine and phosphoserine. This model system suggests that γ-glutamyl transpeptidase inhibition may be a rational approach to alleviating the effects of glutathione synthetase deficiency.

Generalized deficiency of the enzyme glutathione synthetase (E.C. 6.3.2.3.) results in decreased intracellular glutathione content and consequent overproduction of 5-oxoproline (1,2). Glutathione deficiency is associated with oxidant-mediated cell damage, manifested by hemolytic anemia (1,2) and defective polymorphonuclear leukocyte function (3). 5-Oxoprolinuria, with resultant metabolic acidosis, occurs secondary to excess conversion of γ-glutamylcysteine to oxoproline, beyond the capacity of 5-oxoprolinase to convert it to glutamate (1). Inhibition of γ-

*To whom reprint requests should be addressed. Section on Human Biochemical and Developmental Genetics, NICHD, NIH, Bethesda, Maryland 20014

0006-291X/79/140504-08$01.00/0

glutamylcysteine synthetase by cystamine may decrease oxoproline production (4). Ideal therapy, however, would increase cellular glutathione content toward normal, thus decreasing the risk of oxidant damage while increasing feedback inhibition of γ-glutamylcysteine synthetase (5) and secondarily reducing oxoproline production. γ-Glutamyl transpeptidase (EC 2.3.2.1.) is the major catabolic enzyme for glutathione (6) and its inhibition might be expected to increase cellular glutathione content. L-serine, particularly in the presence of borate buffer, is a potent inhibitor of γ-glutamyl transpeptidase (7). Inhibition may result from formation of a serine-borate complex which competes for the γ-glutamyl binding site of the enzyme (8). We therefore examined the effect of serine and borate on cultured skin fibroblasts from a patient with glutathione synthetase deficiency. We have found that exposure to serine-borate produces a significant increase in intracellular glutathione content and an associated decrease in the conversion of ^{14}C-glutamic acid to 5-oxoproline.

MATERIALS AND METHODS

Skin fibroblasts from a patient with glutathione synthetase deficiency and 5-oxoprolinuria (2) were maintained in Eagle's Minimal Essential Medium (N.I.H., #2 Eagle's Basal Medium, equilivant to Eagle's Minimum Essential Medium, Grand Island Biologicals Co., Cat. No. 320-1095, Grand Island, N.Y.) containing 10% fetal calf serum, fresh glutamine, and antibiotics. Studies were performed on confluent cell cultures. L-serine was added to fresh culture medium in isotonic phosphate-buffered saline or sodium borate buffer, pH 7.4, at concentrations indicated. For glutathione determinations, monolayers were rinsed three times with phosphate buffered saline and scraped into 0.02 N HCl. Glutathione was assayed by the method of Tietze (9). Cell protein was determined according to Lowry, et al. (10).

Conversion of ^{14}C-L-glutamic Acid (U) (New England Nuclear Corp.) to 5-oxoproline was examined after 24 hour pretreatment of cells with medium containing 40 mM serine-borate.

The radioactive glutamic acid was repurified on a 0.4 x 6 cm. resin column containing AG-50 (H+) X 8,100-200 mesh (Biorad). The column was washed 3 times with 5 ml. of H_2O to remove any 5-oxoproline, and the glutamate eluted with 2.5 ml of 3N NH_4OH. The eluate was evaporated under nitrogen, resuspended in phosphate buffered saline, and 10μCi added to the medium in each culture dish. Incubation was continued for 18 hours at 37°C. For assay of extracellular 5-oxoproline, medium was aspirated, proteins precipitated with 1/10 volume of 50% trichloroacetic acid, and the supernatant passed over a 0.4 x 12cm AG-50(H+) column. 5-

oxoproline was eluted in 2 X 5 ml. water. Aliquots were mixed with Aquasol (New England Nuclear) and radioactivity determined in a scintillation counter. Intracellular 5-oxoproline was determined after washing the monolayers 3 times with phosphate buffered saline. Cells were scraped into water and 1/10 volume of 50% trichloroacetic acid added. Proteins were removed by centrifugation and quantified by the method of Lowry, et al. (10). ^{14}C-5-oxoproline was isolated and counted as above. Identity of the labelled product from the column was confirmed as oxoproline by high voltage paper electrophoresis in 0.05 N sodium acetate buffer, pH5.5.

γ-Glutamyl transpeptidase activity was assayed with γ-glutamyl-p-nitroanilide in the presence of glycylglycine (11). Glutathione synthetase activity (2) and intracellular amino acid concentrations (12) were determined as previously described.

RESULTS

L-serine inhibited fibroblast γ-glutamyl transpeptidase activity; inhibition was markedly enhanced by borate buffer (Fig. 1). The enzyme was more than 95% inhibited by 20 mM serine and borate. Exposure of

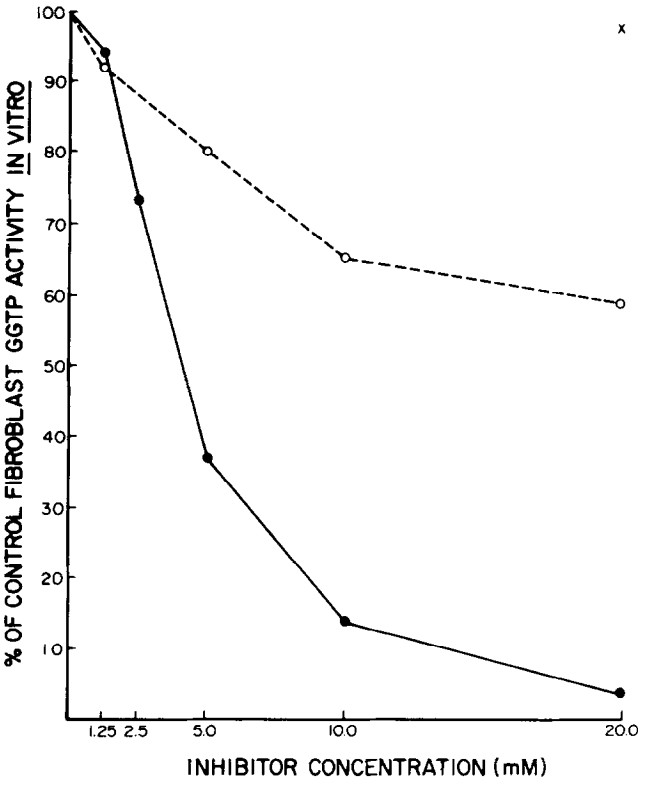

Figure 1. Effect of serine-borate on fibroblast γ-glutamyl transpeptidase (GGTP) activity. Trypsinized fibroblasts were sonicated in 0.1 M Tris and γ-glutamyl transpeptidase assayed at pH 9.0 (11) in the presence of L-serine (0----0), serine borate (●——●), or borate (x).

Table I. Effect of Serine-Borate on the Glutathione Content of Glutathione Synthetase Deficient Fibroblasts*

Cell Line	Treatment	Fibroblast GSH (μg/mg protein)
Normal	0	6.9 ± 0.7 (11)
Glutathione Synthetase Deficient	0	1.9 ± 0.1 (4)
	40 mM serine and borate	4.4 ± 0.3 (6) +
	40 mM serine	2.4 ± 0.1 (6) +
	40 mM borate	1.6 ± 0.2 (6)

*Confluent fibroblasts were incubated in Eagle's MEM with 10% fetal calf serum with addition of the compounds indicated. Borate was added as sodium borate, pH 7.4. After incubation for 24 hours, cells were harvested and glutathione determined (9) as indicated in the text.

+ Different than untreated, $p<0.005$, Student's t-test.

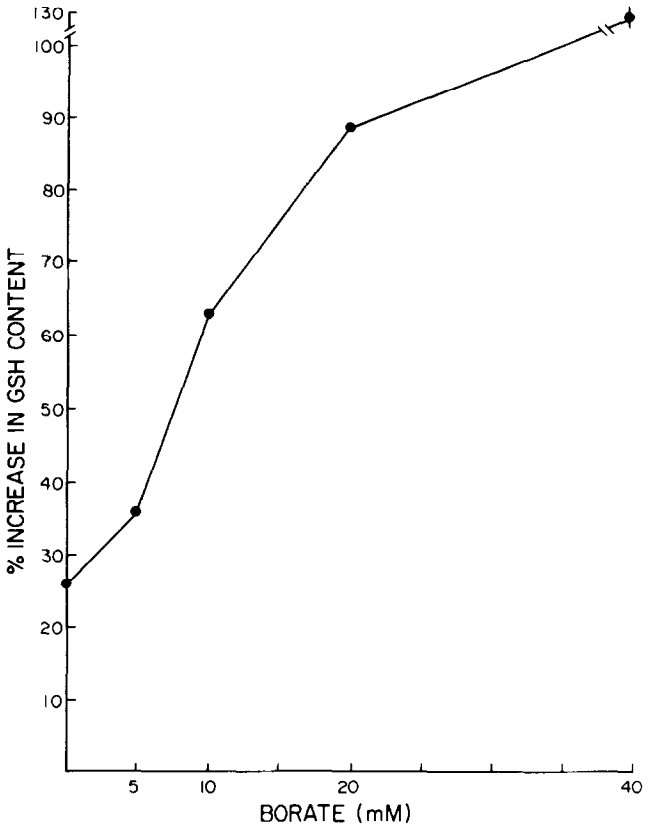

Figure 2. Effect of borate concentration in the presence of 40 mM L-Serine on increase in glutathione synthetase deficient fibroblast glutathione (GSH) content. Experimental conditions as in Table I.

glutathione synthetase deficient fibroblasts to 40mM serine-borate in medium at 37°C for 24 hours produced a more than 2-fold increase in intracellular GSH content (Table 1). Serine alone led to a smaller increase, and borate alone was without effect. Increasing concentrations of borate (up to 40 mM) enhanced the elevation of glutathione by 40 mM serine (Fig. 2). Fibroblast glutathione increased progressively during 6 through 24 hours of treatment with serine-borate (Fig. 3).

Conversion of ^{14}C-L-glutamic acid to 5-oxoproline was increased in glutathione synthetase deficient cells compared to normal when 5-oxoproline was measured both intracellularly and in the medium (Fig. 4). 24 hour pretreatment with serine-borate (40mM) decreased 5-oxoproline

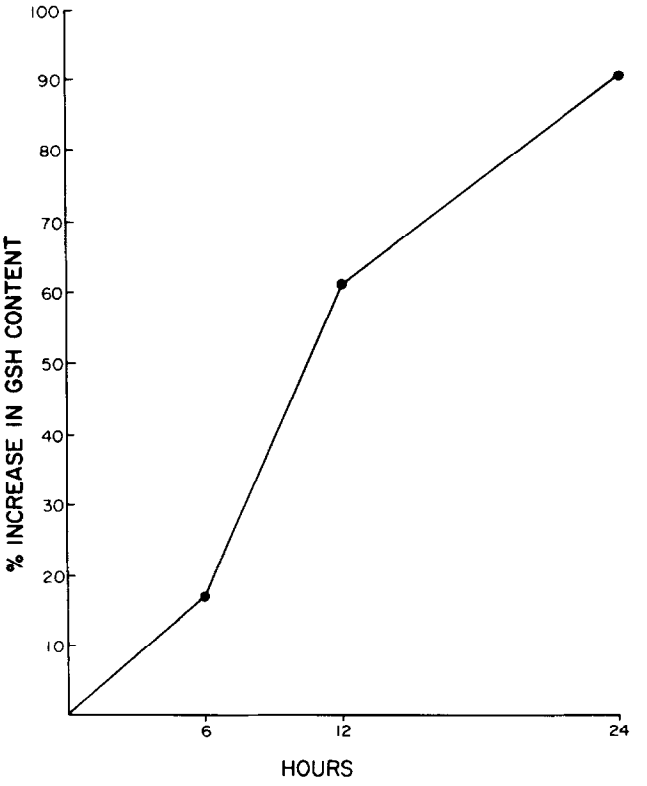

Figure 3. Time course of increase in glutathione (GSH) content of glutathione synthetase deficient fibroblasts on exposure to 40mM serine-borate. Cells were incubated for the indicated periods with medium containing serine-borate and assayed as in Table I.

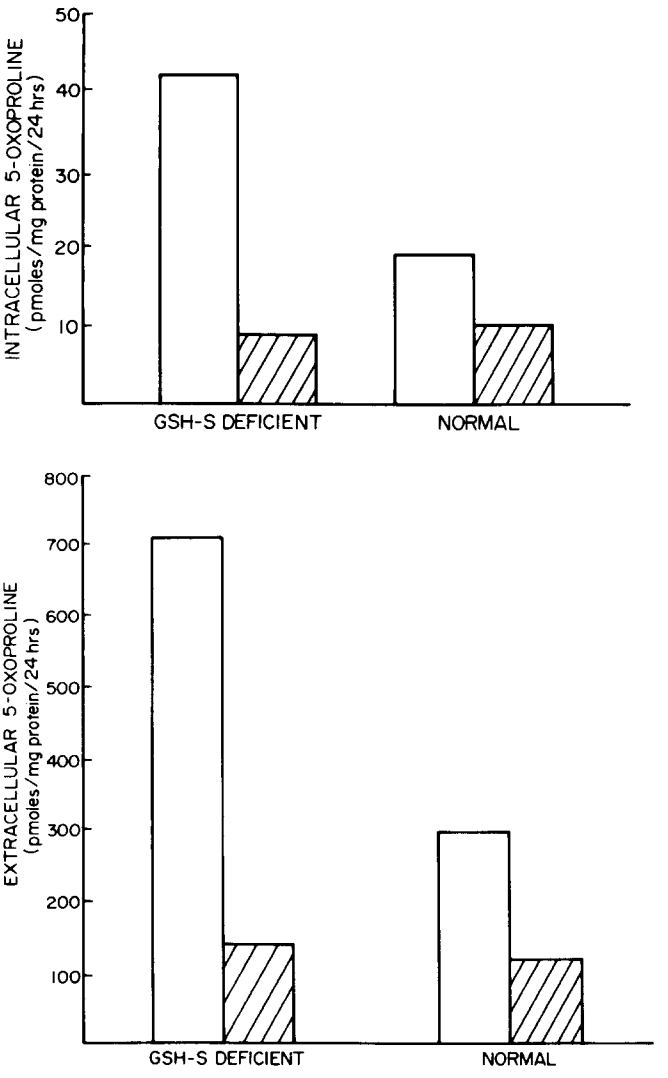

Figure 4. Effect of serine-borate on conversion of ^{14}C-glutamic acid to ^{14}C-5-oxoproline. Experimental conditions are detailed in the text. ☐ = untreated; ▨ = 40 mM serine-borate. GSH-S = Glutathione Synthetase

overproduction in mutant and normal cells; 5-oxoproline production in glutathione synthetase deficient cells was normalized in serine-borate. Under these conditions glutathione synthetase activity in normal or mutant fibroblasts was unchanged.

Intracellular amino acid concentrations were unaltered by 24 hour exposure to medium with 40mM serine-borate except for increased L-serine

(16 ± 3.6 to 67 ± 9.1 nanomoles/mg protein) and phosphoserine (0.7 ± 0.2 to 2.2 ± 0.7 nanomoles/mg protein) concentrations. Cells did not manifest signs of toxicity by light microscopy during serine-borate addition to the medium.

DISCUSSION

The clinical signs and symptoms of glutathione synthetase deficiency result both from decreased intracellular glutathione content and 5-oxoproline overproduction. Rational therapy thus should be aimed at correction of both biochemical abnormalities. Inhibition of γ-glutamylcysteine synthetase decreases 5-oxoproline formation (4) but should further reduce glutathione synthesis. Our previous studies have shown that the antioxidant α-tocopherol can partially correct the hemolytic anemia (13) and leukocyte function defects (14) associated with decreased intracellular glutathione secondary to glutathione synthetase deficiency. The present studies demonstrate that inhibition of glutathione catabolism by inhibiting γ-glutamyl transpeptidase with serine-borate increases cell glutathione content as well as decreasing 5-oxoproline production. The advantage of such an approach to therapy is that cells would be protected from oxidant damage, and metabolic acidosis and other possible toxicity from 5-oxoproline would be diminished.

Inhibition of γ-glutamyl transpeptidase by serine-borate has been reported to increase rat renal glutathione content *in vivo* (15). Borate is toxic (16) and the long-term effects of γ-glutamyl transpeptidase inhibition are unknown. It is possible that safe inhibitors could be developed which might be therapeutically useful in glutathione synthetase deficiency, and the use of such inhibitors in tissue culture may provide a useful tool for exploring the roles of glutathione and 5-oxoproline in cellular metabolism.

The investigations reported here document the correction *in vitro* by serine-borate of the two major metabolic consequences of glutathione

synthetase deficiency (low cellular glutathione and oxoproline overproduction). This approach represents a model for therapy of inborn errors of metabolism - inhibition of a "normal" enzyme to compensate for the consequences of a genetically determined deficiency in activity of another enzyme. This concept may merit consideration with regard to certain other heritable disorders.

ACKNOWLEDGEMENTS

This work was carried out in part in the Burroughs-Welcome Developmental Pharmacology Laboratory at The Johns Hopkins Hospital and was supported in part by N.I.H. general research support grant No. RR-5378 (SPS). We wish to thank Barbara Foley and Louise M. Schulden for expert Technical Assistance and Mrs. Penny Colbert and Ms. Dana Jarvis for typing the manuscript.

REFERENCES

1. Wellner, V.P., Sekura, R., Meister, A., and Larsson, A., Proc. Natl. Acad. Sci. U.S.A., 71, 2505-2509 (1974).
2. Spielberg, S.P., Kramer, L. I., Goodman, S.I., Butler, J., Tietze, F., Quinn, P., and Schulman, J.D., J. Pediat., 91, 237-241 (1977).
3. Spielberg, S.P., Boxer, L.A., Oliver, J.M., Allen, J.M., and Schulman, J.D., Brit. J. Haematol., in press.
4. Griffith, O.W., Larsson, A., and Meister, A., Biochem. Biophys. Res. Comm., 79, 919-925 (1977).
5. Richmond, P., and Meister, A., J. Biol. Chem., 250, 1422-1426 (1975).
6. Meister, A., Science, 180, 33-39 (1973).
7. Revel, J.P., and Ball, E.G., J. Biol. Chem., 234, 577-582 (1959).
8. Tate, S.S., and Meister, A., Proc. Natl. Acad. Sci. U.S.A., 75, 4806-4809 (1978)
9. Tietze, F., Anal. Biochem., 27, 502-522 (1969).
10. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J., J. Biol. Chem., 193, 265-275 (1951).
11. Orlowski, M., and Meister, A., Biochim. Biophys. Acta, 73, 679-681 (1963).
12. Schneider, J.A., Rosenbloom, F.M., Bradley, K.H. and Seegmiller, J.E., Biochem. Biophys. Res. Comm., 29, 527-53 (1967).
13. Spielberg, S.P., Boxer, L.A., Corash, L.M., and Schulman, J.D., Ann. Intern. Med., 90, 53-54 (1979).
14. Boxer, L. A., Oliver, J. M., Spielberg, S.P., Allen, J.M., and Schulman, J.D., Blood, 52, Supp. 1, 145 (1978).
15. Griffith, O.W., Bridges, R.J., and Meister, A., Proc. Natl. Acad. Sci. U.S.A., 75, 5405-5408 (1978).
16. Valdes-Dapena, M.A., and Arey, J.B., J. Pediat., 61, 531-546 (1962).