Vol. 127, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1985 Pages 270-276

ENHANCEMENT OF INTRACELLULAR GLUTATHIONE PROTECTS ENDOTHELIAL CELLS AGAINST OXIDANT DAMAGE

Min-Fu Tsan, Ellen H. Danis,
Peter J. Del Vecchio and Carmen L. Rosano

Research Service, Albany Veterans Administration Medical Center,
Departments of Physiology, Medicine and Ophthalmology,
Albany Medical College of Union University
Albany, New York 12208

Received January 11, 1985

We studied the role of glutathione in the endothelial cell defense against H_2O_2 damage. Treatment of endothelial cells with buthionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase, depleted the cells of GSH, while L-2-oxothiazolidine-4-carboxylate, an effective intracellular cysteine delivery agent, markedly enhanced endothelial cell GSH concentration. Depletion of intracellular GSH sensitized the endothelial cells to injury by H_2O_2 either preformed or generated by the glucose-glucose oxidase system. In contrast, an increase of intracellular GSH protected the cells from H_2O_2 damage. There was an inverse, linear relationship between the intracellular GSH concentrations and killing of endothelial cells by H_2O_2. Our results suggest that enhancement of endothelial cell GSH may be an alternative approach toward the prevention of oxidant-induced endothelial damage such as adult respiratory distress syndrome.

Glutathione (GSH) plays an important role in protecting cells against the destructive effects of reactive oxygen intermediates and free radicals (1). Depletion of GSH of tumor cells increases their sensitivity to irradiation (2,3) or oxidant damage mediated by activated neutrophils or macrophages as well as glucose-glucose oxidase system (4). Inhibition of GSH synthesis also augments lysis of murine tumor cells by sulfhydryl-reactive antineoplastics (5). Thus, useful effect may be achieved by depletion of GSH. The physiological function of GSH in protecting cells against reactive oxygen intermediates and free radicals suggests that an increase in cellular GSH may also be beneficial under certain conditions.

Recent advances in our knowledge of the synthesis and metabolism of GSH make it possible to modulate the intracellular level of GSH (1). In this study, we demonstrated that treatment of endothelial cells in culture with buthionine

0006-291X/85 $1.50

sulfoximine (BS), an irreversible inhibitor of γ-glutamylcysteine synthetase (6), depleted the cells of GSH, while L-2-oxothiazolidine-4-carboxylate (OTC), an effective intracellular cysteine delivery agent (7), markedly enhanced endothelial cell GSH concentrations. Depletion of intracellular GSH sensitized the endothelial cells to injury by H_2O_2. In contrast, an increase of intracellular GSH protected the cells from H_2O_2 damage.

MATERIALS AND METHODS

L-2-oxothiazolidine-4-carboxylate (OTC) was synthesized by the method of Kaneko et al (8) with a slight modification. The modification consisted of isolating the product by repeated extraction of the acidified aqueous solution with ethyl acetate. Our product had a melting point of 171°C. Element analysis revealed: C, 32.60%; H, 3.26%; N, 9.48%; S, 21.48% (calculated values for $C_4H_5NO_3S$: C, 32.64%; H, 3.42%; N, 9.52%; S, 21.79%). The sodium salt of OTC was used throughout the study.

DL-buthionine-S-R-sulfoximine (BS) was obtained from Chemalog, South Plainfield, NJ. Glutathione, GSH reductase (Type IV, EC 1.6.4.2), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 5,5'-dithiobis-2-nitrobenzoate (DTNB) and glucose oxidase (Type VII, EC 1.1.3.4) were purchased from Sigma Chemical Co., St. Louis, MO. [^{51}Cr]-sodium chromate was obtained from Amersham Corporation, Arlington Heights, Ill., and H_2O_2 (30%) was obtained from Fisher Scientific Co., Fairlawn, N.J.

Calf pulmonary artery endothelial cells were isolated as described previously (9). They were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, New York) supplemented with 20% donor calf bovine serum (Flow Lab Inc., McLean, VA) and garamycin (50 μg/ml, Schering Corp., Kenilworth, N.J.) in Corning 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, New York). The cells were always more than 95% viable as judged by their exclusion of trypan blue. The endothelial nature of the cells has been established by the presence of factor VIII related antigen, angiotensin-converting enzyme activity and Weibel-Palade Bodies (9).

For the measurement of endothelial cell GSH concentrations, confluent endothelial cell monolayers in Corning 35 mm tissue culture dishes [$(4.1\pm0.7) \times 10^5$ cells/dish, n=7] were used. They were incubated with OTC and/or BS in 2 ml DMEM containing 20% donor calf serum at 37° in a 5% CO_2 incubator for 16 hours unless otherwise indicated. After incubation, cell monolayers were washed twice with 2 ml phosphate buffered saline (pH 7.4) and extracted according to Burchill et al (10). Glutathione concentration in the cell extracts was then determined by the method of Tietze (11).

Cytotoxicity was determined by [^{51}Cr]-release assay (12). Endothelial cell monolayers in Corning multiwell (16 mm in diameter) tissue culture plates were incubated with OTC (1 mM) and/or BS (0.04 mM) in 0.5 ml DMEM containing 20% calf serum and 1 μCi [^{51}Cr]-sodium chromate for 16 hours at 37° C in a 5% CO_2 incubator. After washing twice with 1 ml phosphate buffered saline, the cells were incubated with 0.5 ml Earle's balanced salt solution containing 5 mM glucose, 0.25% calf serum and various amounts of glucose oxidase or H_2O_2, for 3 hours at 37° C. Under this condition, one unit of glucose oxidase generated 0.37 ± 0.04 μmole H_2O_2 per minute (n=4); H_2O_2 production was determined by the disappearance of scopoletin fluorescence in the presence of horseradish peroxidase as described previously (13). The supernate was removed and centrifuged in a microcentrifuge at 13,000 rpm for 3 min at room temperature. The radioactivity in 0.2 ml of the supernates was

determined by counting in a well scintillation gamma counter (Ortec Comp., Oak Ridge, TN). The maximum releasable radioactivity was determined by incubating the endothelial cell monolayer with 0.5 ml of Earle's Balanced Salt Solution containing 1% Triton X-100 as described above and the amount of radioactivity in the supernatant was determined. Cytotoxicity was expressed as the percentage of [^{51}Cr] release calculated by the formula (14):

$$\text{Percent killing} = \frac{A-B}{C-B} \quad X100$$

where A was the radioactivity in the supernate of samples containing glucose oxidase or H_2O_2 and endothelial cells, B was the radioactivity in the supernate of samples containing endothelial cells alone, and C was the maximum releasable radioactivity of the endothelial cells.

RESULTS

L-2-oxothiazolidine-4-carboxylate has been shown to markedly enhance the hepatic GSH level in mice (7,15). We studied its effect on the GSH levels of cultured endothelial cells. When the cells were incubated with OTC overnight (16 hours), there was an increase in the intracellular GSH concentration. In addition, there was a dose-dependent response of intracellular GSH to increasing concentrations of OTC (Figure 1a). It approached a plateau at an OTC concentration of 1 mM; thereafter, further increasing the OTC concentrations only brought about a slight increase in the GSH level. Figure 1b is a time course experiment. Using 1 mM of OTC, there was a 38% stimulation of GSH concentration at 4 hours after incubation, whereas at 16 hours after incubation, there was a 50% stimulation.

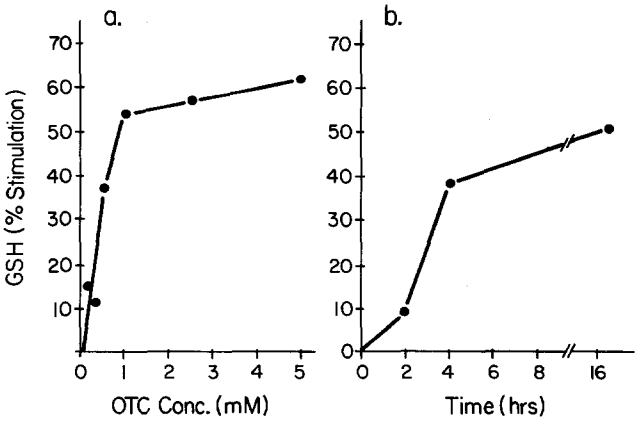


Figure 1: Effect of OTC on intracellular GSH of endothelial cells: a) dose response experiment, b) time course experiment. Endothelial cell monolayers were incubated with various amounts of OTC for 16 hours at 37° C (a) or with 1 mM of OTC for various intervals at 37° C (b). After incubation, the cells were extracted and GSH concentrations determined. The results were expressed as percent stimulation over control. Each point was the mean of 2 or 3 experiments.

TABLE 1. EFFECT OF OTC ON INTRACELLULAR GSH OF ENDOTHELIAL CELLS

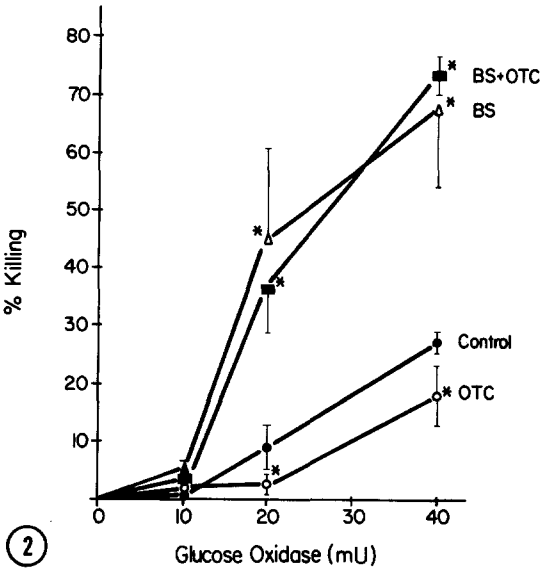
	GSH (nmoles/10^6cells)	Percent Control	p values (vs control)
Control	6.61±1.07 (14	(100)	
OTC (1mM)	9.80±1.82 (14)	148.0	<0.005
BS (0.04 mM)	1.82±0.96 (5)	27.5	<0.01
OTC (1mM)+BS (0.04mM)	2.28±1.33 (6)	34.4	<0.001

Endothelial cell monolayers were incubated in the presence or absence of OTC and/or BS for 16 hours at 37°C. The cells were then extracted and GSH concentration determined. The results were expressed as nmoles GSH/10^6 cells (mean±SEM). Numbers in parentheses indicated the number of experiements. p values were calculated using Student's t test.

Based on the above observation, our further experiments were done with 1 mM OTC and 16 hours of incubation.

It has been suggested that OTC is an effective intracellular delivery agent of cysteine, which in turn is rapidly utilized by γ-glutamycysteine synthetase for the synthesis of GSH (1,7,16). If this were the case, then the effect of OTC on intracellular GSH should be inhibited by BS, an irreversible inhibitor of γ-glutamylcysteine synthetase (6). As shown in Table 1, BS markedly suppressed the level of GSH in endothelial cells. In addition, it abolished the stimulatory effect of OTC; the concentrations of GSH in endothelial cells were similar when cells were incubated with BS in the presence or absence of OTC.

Since one major function of GSH is the protection of cells against oxidant damage, we then determine whether increasing the intracellular GSH concentration can enhance the protection of cells. We used H_2O_2 either preformed or generated by the glucose-glucose oxidase system as the oxidant, because these systems have been shown to damage endothelium (14,17). As shown in Figure 2, pretreatment with OTC which enhanced endothelial cell GSH concentrations, reduced endothelial cytolysis by the glucose-glucose oxidase system. In contrast, depletion of GSH by BS, markedly enhanced endothelial cell killing by the glucose-glucose oxidase system. Similar results were obtained when H_2O_2 was used (0.2 mM H_2O_2 6 experiments, data not shown). The protection of endothelial cell damage by OTC was not due to



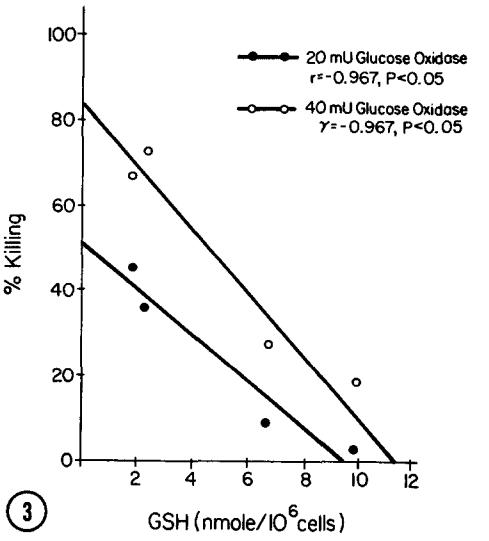


Figure 2: Killing of endothelial cells by glucose oxidase. Endothelial cells were pretreated with OTC (1 mM) and/or BS (0.04 mM), and labeled with ^{51}Cr for 16 hours at 37° C. They were then exposed to various amounts of glucose oxidase for 3 hours at 37° C. The release of ^{51}Cr from ^{51}Cr-endothelial cells was used as an index of cell-killing. Each point was the result (mean±SEM of percent killing) of 4 experiments. * denotes p value (vs control) of <0.05.

Figure 3: Correlation between endothelial cell GSH concentration and killing by glucose-oxidase. Data points were derived from Table 1 and Figure 2. Curves were obtained by least-squares method of linear regression. γ = correlation coefficient. p values were obtained by two-tailed t test.

its non-specific effects, since OTC did not protect BS-treated cells. Furthermore, there was an inverse, linear relationship between the intracellular GSH concentration and percent killing of endothelial cells by H_2O_2 (Figure 3), with a correlation coefficient of -0.967 ($p<0.05$).

DISCUSSION

The results presented demonstrate that L-2-oxothiazolidine-4-carboxylate can stimulate the intracellular concentration of GSH in endothelial cells. The enhanced concentration of GSH offers additional protection to cells against H_2O_2 induced injury to endothelium. Recently, Wellner et al (18) also demonstrate that enhancement of intracellular GSH of human lymphoid cell lines by GSH ester, protects cells against the lethal effect of irradiation. In contrast, depletion of intracellular GSH by buthionine sulfoximine sensitized the endothelial cells

to injury by H_2O_2. This latter observation has been previously reported by Harlan et al (19).

L-2-oxothiazolidine-4-carboxylate is an analog of 5-oxoproline (16). It is converted by 5-oxoprolinase to S-carboxy-L-cysteine which breaks down spontaneously to L-cysteine (1,7). Administration of OTC, either parenterally or orally, to mice causes a dramatic increase (more than 100%) of hepatic GSH and protects the animals from acetaminophen toxicity (7,15). Thus, OTC appears to be an effective intracellular delivery agent of cysteine for the synthesis of GSH. Our results extend the above observation of Williamson and Meister (7,15) and demonstrate that OTC can also enhance cellular GSH levels in a tissue culture system.

Our observation that enhanced GSH concentration protects endothelial cells against H_2O_2-induced damage is of potential importance. Endothelial cells play an essential role in maintaining vascular integrity. Acute endothelial damage leading to pulmonary edema appears to be an initial event in the development of adult respiratory distress syndrome (ARDS) (20,21). There is considerable clinical and experimental evidence to suggest that this endothelial damage is mediated by oxidant produced by activated neutrophils (20-22). A variety of insults that predispose patients to ARDS, activate complement. Complement activation generates C_{5a}, which aggregates and stimulates neutrophils leading to pulmonary leukostasis and acute edematous lung injury (20-22). In vitro studies (14,17,23,24) reveal that endothelial damage by activated neutrophils is mediated by H_2O_2. Our results suggest that enhancement of intracellular GSH by OTC may protect endothelium against neutrophil damage and may provide an alternative approach toward the prevention of ARDS.

ACKNOWLEDGEMENT

This work is supported by the Veterans Administration Research Service and a grant from the U.S. Public Health Service PO1 AG-03275. The secretarial work of Mary Peplowski and Marie Stoddard is appreciated.

REFERENCES

1. Meister, A. (1983) Science 220:472-477.

2. Dethmers, J.K., and Meister, A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:7492-7496

3. Mitchell, J., Russo, A., Biaglow, J., and McPherson, S. (1983) Radiat. Res. 96:422-428.

4. Arrick, B.A., Nathan, C.F., Griffith, O.W., and Cohn, Z.A. (1982) J. Biol. Chem. 257:1231-1237.

5. Arrick, B.A., Nathan, C.F., and Cohn, Z.A. (1983) J. Clin. Invest. 71:258-267.

6. Griffith, O.W., and Meister, A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76:268-272.

7. Williamson, J.M., Boettcher, B., and Meister, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79:6246-6249.

8. Kaneko, T., Shimokobe, T., Ota, Y., Toyokawa, E., Inui, T., and Shiba, T. (1964) Bull. Chem. Soc. Jpn. 37:242-244.

9. Del Vecchio, P.J., and Smith, J.R. (1981) J. Cellul. Physiol. 108:337-345.

10. Burchill, B.R., Oliver, J.M., Pearson, C.B., Leonbach, E.D., and Berlin, R.D. (1978) J. Cell. Biol. 76:439-447.

11. Tietze F. (1969) Anal. Biochem. 27:502-522.

12. Tsan, M.F. (1980) J. Cellul. Physiol. 105:327-334.

13. Tsan, M.F., Douglass, K.H., and McIntyre, P.A. (1977) Blood 49:439-444.

14. Weiss, S.J., Young, J., LoBuglio, A.F., Slivka, A., and Nimeh, N.F. (1981) J. Clin. Invest. 68:714-721.

15. Williamson, J.M., and Meister, A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:936-939

16. Williamson, J.M., and Meister, A. (1982) J. Biol. Chem. 257:12039-12042.

17. Harlan, J.M., and Callahan, K.S. (1984) J. Clin. Invest. 74:442-448.

18. Wellner, V.P., Anderson, M.E., Puri, R.N., Jensen, G.L., and Meister, A. (1984) Proc. Natl. Acad. Sci. U.S.A. 81:4732-4735.

19. Harlan, J.M., Levine, J.D., Callahan, K.S., Schwartz, B.R., and Harker, H.A. (1984) J. Clin. Invest. 73:706-713.

20. Rinaldo, J.E., and Rogers, R.M. (1982) N. Engl. J. Med. 306:900-909.

21. Tate, R.M., and Repine, J.E. (1983) Am. Rev. Respir. Dis. 128:552-559.

22. Craddock, P.R., Fehr, J., Brigham, K.L., Kronenberg, R.S., and Jacob, H.S. (1977) N. Engl. J. Med. 296:769-774.

23. Sacks, T., Moldon, C.F., Craddock, P.R., Bowers, T.K., and Jacob, H.S. (1978) J. Clin. Invest. 61:1161-1167.

24. Suttorp, N., and Simon, L.M. (1982) J. Clin. Invest. 70:342-350.