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Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis

(L-2-oxothiazolidine-4-carboxylate/liver/acetaminophen/parenteral therapy/safener)

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ABSTRACT **Depletion of glutathione by inhibition of its synthesis by buthionine sulfoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase, leads to increased sensitivity to (*i*) irradiation and (*ii*) oxidative stress. In the present work, an intracellular cysteine delivery system was used to promote glutathione synthesis, and this was found to protect against toxicity. Thus, administration of L-2-oxothiazolidine-4-carboxylate protected against acetaminophen toxicity in mice; the thiazolidine, which is converted to L-cysteine by the enzyme 5-oxo-L-prolinase (present in many animal tissues and in plants) promotes the synthesis of glutathione, which is the actual protectant. The effect of this thiazolidine in increasing the level of glutathione is prevented by administration of buthionine sulfoximine. This thiazolidine may be useful in the treatment of other toxicities and in the treatment of certain diseases. It may also be valuable as a component of amino acid mixtures used in therapy and as a safener in agriculture.**

Recent studies in this laboratory have shown that it is possible to modulate the synthesis and metabolism of glutathione by administration of selective enzyme inhibitors (1). Thus, inhibition of γ-glutamylcysteine synthetase by administration of buthionine sulfoximine to animals (2, 3) or to cells grown in tissue culture (4) leads to a substantial decline in the intracellular glutathione concentration. Decreased glutathione synthesis has been found to have the following effects: decreased cell viability (4), increased sensitivity of cells to the effects of irradiation (4), increased sensitivity of tumor cells to cytolysis by peroxide (5) (see also ref. 6), decreased synthesis of prostaglandin E and leukotriene C (7), and selective destruction of trypanosomes in mice (8). Although these effects of glutathione depletion are clearly of interest, the possibility that an increase in tissue or cellular glutathione might lead to potentially useful effects also needs to be considered. That the glutathione content of tissues may be increased by supplying certain precursors of this tripeptide has been indicated by studies showing that administration of γ-glutamylcysteine and related compounds to mice leads to increased levels of renal glutathione (9) and that administration of L-2-oxothiazolidine-4-carboxylate to mice produces a substantial increase in liver glutathione levels (10).

In the present work, we have examined the use of L-2-oxothiazolidine-4-carboxylate in a cysteine delivery system that protects mice against the toxic effects of acetaminophen. The biochemical basis of this effect lies in the fact that this thiazolidine is an excellent substrate of the enzyme 5-oxo-L-prolinase, which converts this substrate to *S*-carboxy-L-cysteine, which spontaneously decarboxylates to yield L-cysteine. The L-cysteine formed in this manner is rapidly utilized for glutathione synthesis. Since 5-oxo-L-prolinase is found in many animal tissues, the thiazolidine is probably utilized for glutathione synthesis in tissues other than the liver, and therefore its administration to animals would serve as a cysteine delivery system for many organs. The present studies suggest that administration of this thiazolidine may be effective (*i*) in protecting against other toxic compounds (including certain antitumor drugs), (*ii*) as a component of amino acid mixtures used for oral and parenteral administration, (*iii*) for treatment of individuals who have diminished utilization of methionine sulfur for cysteine formation, and (*iv*) as a safener in agriculture to protect plants against toxic effects of herbicides.

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EXPERIMENTAL PROCEDURES

L-2-Oxothiazolidine-4-carboxylate (10), DL-buthionine-*SR*-sulfoximine (2), diethyl maleate, and acetaminophen (Sigma) were obtained as indicated. Mice (NCS strain; 15–25 g) were obtained from the Rockefeller University. The animals, fasted overnight, were injected with various compounds as described below and were killed by decapitation and then exsanguinated. Liver samples were homogenized in ice-cold 1% picric acid (5 vol/g of tissue). After homogenization, insoluble material was removed by brief centrifugation at low speed, and the protein-free supernatant solutions were analyzed (in duplicate) for glutathione (11). In the experiments described in Tables 1, 2, and 3, four mice were used in each experiment and the values are expressed as mean ± SD.

RESULTS

Mice exhibited a marked decrease in liver glutathione levels 4 hr after treatment with diethylmaleate or acetaminophen (Table 1). When L-2-oxothiazolidine-4-carboxylate was given together with either diethyl maleate or acetaminophen, the liver glutathione levels were substantially higher (about 2.5-fold) than the controls. This result is similar to that found in studies with untreated mice (10). Treatment of mice with buthionine sulfoximine also led to a marked depression of liver glutathione levels [in agreement with earlier findings (2, 3)], but mice treated with both buthionine sulfoximine and the thiazolidine had about the same low level of liver glutathione. This finding is to be expected because buthionine sulfoximine inhibits γ-glutamylcysteine synthetase (2, 3).

The effect of administration of L-2-oxothiazolidine-4-carboxylate before or at various times after acetaminophen administration is described in Table 2. It is of interest that the thiazolidine produced a substantial effect on the liver glutathione level even when it was given 2 hr after acetaminophen administration. The effects of several different dosages of the thiazolidine (given 2 hr after acetaminophen) on liver glutathione are described in Table 3. Under the conditions used, a considerable increase in liver glutathione levels was observed after doses varying from 1 to 6.5 mmol/kg were given.

Table 1. Effect of L-2-oxothiazolidine-4-carboxylate on liver reduced glutathione (GSH) levels after administration of DL-buthionine-*SR*-sulfoximine, diethyl maleate, or acetaminophen

Sample	GSH, μmol/g of tissue	% control
Control	3.2 ± 0.3	100
Diethyl maleate	1.9 ± 0.1	59
Diethyl maleate; L-2-oxothiazolidine-4-carboxylate	8.2 ± 0.4	260
Acetaminophen	0.68 ± 0.13	21
Acetaminophen; L-2-oxothiazolidine-4-carboxylate	7.6 ± 0.4	240
DL-Buthionine-*SR*-sulfoximine	0.88 ± 0.13	28
DL-Buthionine-*SR*-sulfoximine; L-2-oxothiazolidine-4-carboxylate	0.83 ± 0.08	27

DL-Buthionine-*SR*-sulfoximine (4 mmol/kg), diethyl maleate (in sesame oil) (2 mmol/kg), and acetaminophen (2.5 mmol/kg) were administered intraperitoneally. A neutral solution of sodium L-2-oxothiazolidine-4-carboxylate (8 mmol/kg) was given, as indicated, subcutaneously 2 min later. Four hours later, liver GSH levels were determined.

In the experiment described in Fig. 1, mice were injected with acetaminophen and either *N*-acetyl-L-cysteine or L-2-oxothiazolidine-4-carboxylate. The subsequent time courses of the liver glutathione levels indicate that both compounds produce a substantial increase in glutathione levels and that the effect of the thiazolidine is greater than that of *N*-acetyl-L-cysteine.

In the experiment described in Fig. 2, five groups of mice were treated orally with acetaminophen (2.5 mmol/kg); after 1 hr, four of the groups were treated orally with different doses of the thiazolidine as indicated. Although no increase in 7-day survival over the control was found when the thiazolidine was given at 2.5 mmol/kg, survival increased progressively with increases in the dose of thiazolidine and 100% protection was observed with a dose of 20 mmol/kg.

DISCUSSION

Previous studies have shown that the toxicity of acetaminophen involves its conversion to a highly reactive intermediate that interacts rapidly with various cell components including glutathione (12–16). Acetaminophen is also detoxified to some extent by other pathways. Toxicity due to acetaminophen is ameliorated by administration of *N*-acetyl-L-cysteine and it appears that this compound is deacetylated *in vivo*. Although the site or sites of this reaction have apparently not yet been extensively examined, it is known that kidney and to lesser extents liver and other tissues exhibit amino acid *N*-acylase activity (17). The present findings indicate that L-2-oxothiazolidine-4-carboxylate is probably more effective than *N*-acetyl-L-cysteine in protecting against acetaminophen toxicity. Administered cysteine is relatively ineffective in protecting against acetaminophen toxicity probably, at least in part, because cysteine is rapidly metabolized. It is notable that cysteine itself exhibits toxicity. Thus, cysteine administration has been reported to produce central nervous system damage (18, 19) and addition of excess

Table 2. Effect of time of administration of L-2-oxothiazolidine-4-carboxylate on liver GSH levels after administration of acetaminophen

Sample	GSH, μmol/g of tissue	% control
Control	4.2 ± 0.7	100
Acetaminophen	0.74 ± 0.3	18
L-2-Oxothiazolidine-4-carboxylate	8.9 ± 0.8	210
Acetaminophen; prior administration of L-2-oxothiazolidine-4-carboxylate (6.5 mmol/kg)	6.9 ± 0.8	160
Acetaminophen; subsequent administration of L-2-oxothiazolidine-4-carboxylate:		
30 min later	6.1 ± 0.2	150
60 min later	6.6 ± 0.9	160
120 min later	5.3 ± 1.0	130

Acetaminophen (2.5 mmol/kg) was administered to mice intraperitoneally. A neutral solution of sodium L-2-oxothiazolidine-4-carboxylate (6.5 mmol/kg) was given subcutaneously 5 min before acetaminophen injection or 30, 60, or 120 min, as indicated, after the acetaminophen injection. Four hours after administration of acetaminophen, liver GSH levels were determined.

Table 3. Effect of L-2-oxothiazolidine-4-carboxylate on liver GSH levels after administration of acetaminophen

Sample	GSH, μmol/g of tissue	% control
Control	3.7 ± 0.7	(100)
Acetaminophen	1.6 ± 0.3	43
Acetaminophen; L-2-oxothiazolidine-4-carboxylate (1.0 mmol/kg)	5.7 ± 1.0	150
Acetaminophen; L-2-oxothiazolidine-4-carboxylate (3.0 mmol/kg)	7.5 ± 0.5	200
Acetaminophen; L-2-oxothiazolidine-4-carboxylate (6.5 mmol/kg)	7.0 ± 0.5	190

Acetaminophen (2.5 mmol/kg) was administered to mice intraperitoneally. Two hours later, a neutral solution of sodium L-2-oxothiazolidine-4-carboxylate was given subcutaneously. Four hours after administration of acetaminophen, liver GSH levels were determined.

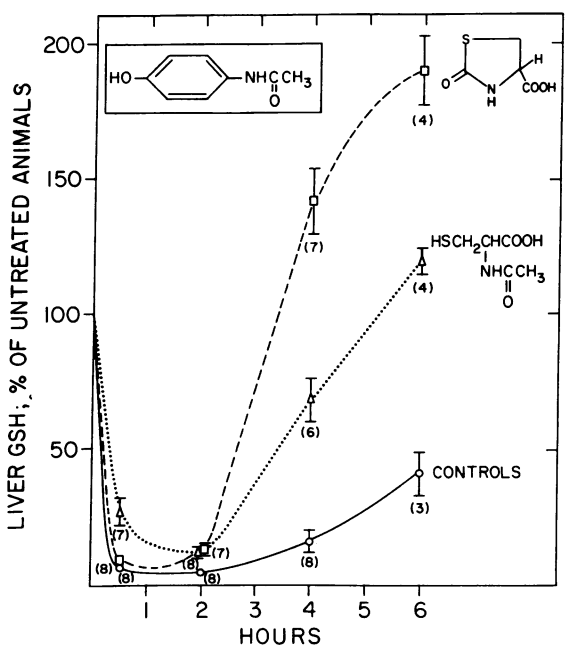

FIG. 1. Effects of L-2-oxothiazolidine-4-carboxylate and *N*-acetyl-L-cysteine on liver glutathione levels in mice treated with acetaminophen. Animals were injected intraperitoneally with acetaminophen (*Inset*) at 2.5 mmol/kg; 5 min later, one group was injected with the thiazolidine at 2 mmol/kg and another group was injected with *N*-acetyl-L-cysteine at 2 mmol/kg. The animals were sacrificed at the indicated intervals. Numbers in parentheses are numbers of animals used for the indicated time point. Results are means ± range; points given without bars had ranges of ±10%.

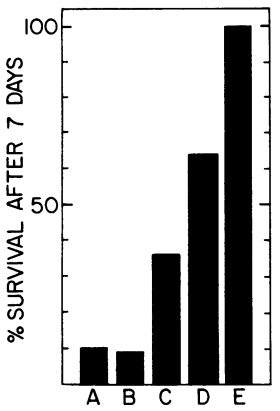

FIG. 2. Survival of mice given a lethal (LD_{90}) dose of acetaminophen; effect of subsequent administration of L-2-oxothiazolidine-4-carboxylate. Mice were given acetaminophen at 9.5 mmol/kg orally, and the thiazolidine was given orally 1 hr later. Bars: A, controls (untreated); B, thiazolidine at 2.5 mmol/kg; C, thiazolidine at 5 mmol/kg; D, thiazolidine at 10 mmol/kg; E, thiazolidine at 20 mmol/kg. Each group consisted of 12–15 mice.

cysteine to the diets of rodents has been reported to lead to death (20).

It seems unlikely that intracellular cysteine itself provides substantial protection against acetaminophen and similar compounds because the tissue levels of cysteine are exceedingly low, even after administration of the thiazolidine. The available data thus indicate that a substantial level of glutathione is an important factor in combating such toxicity and suggest that a readily available supply of cysteine for glutathione synthesis is crucial for protection.

It is now recognized that glutathione is involved in the detoxification of many foreign compounds by pathways initiated by their conjugation with the sulfhydryl moiety of this tripeptide. It is likely that L-2-oxothiazolidine-4-carboxylate would also be useful in the treatment of certain other types of toxicities associated with poisoning and with certain types of therapy, such as anticancer treatments. Therapies in which quantitative differences between the 5-oxoprolinase and glutathione S-transferase activities of tumors and normal tissues are exploited should be considered.

The present findings and previous work (10) suggest that L-2-oxothiazolidine-4-carboxylate may be useful as a component of amino acid mixtures used in diets and in solutions used for parenteral administration. At present most such preparations do not contain cysteine; addition of cysteine may be expected to be associated with complications due to its toxicity and to its oxidation to cystine. Amino acid mixtures containing L-2-oxothiazolidine-4-carboxylate may be useful for patients who are not able to consume a regular diet and who may be deficient in transsulfuration and thus not able to use methionine sulfur for cysteine formation by the cystathionine pathway effectively. There is evidence that the cystathionine pathway is reduced or absent in fetal tissues and in the neonatal period (21, 22). Utilization of methionine sulfur for cysteine synthesis may be greatly reduced in certain inborn errors of metabolism such as hereditary tyrosinosis (23). It is also reduced in liver disease (24); since the liver is the principal site of transsulfuration, an exogenous source of cysteine is of importance for such individuals. It should be noted that conversion of L-2-oxothiazolidine-4-carboxylate to L-cysteine is catalyzed by an enzyme found in almost all tissues; the erythrocyte and ocular lens are exceptions (25).

It has been reported that certain cells grown in tissue culture are highly sensitive to the toxic effect of cysteine (26); inclusion of L-2-oxothiazolidine-4-carboxylate in tissue culture media might obviate such difficulties.

The principles involved in the protection of animals against foreign compounds by L-2-oxothiazolidine-4-carboxylate are probably also applicable to plants, which are known to contain 5-oxoprolinase (27). Thus, it is possible that this thiazolidine may be useful as a safener in agriculture to protect crop plants against herbicides.

There are other ways in which cysteine might be delivered intracellularly for glutathione synthesis. Thus, γ-glutamylcysteine, γ-glutamylcystine, and γ-glutamylcysteine disulfide increase renal glutathione levels (9) by by-passing the feedback-inhibited step of glutathione synthesis (28)—i.e., that catalyzed by γ-glutamylcysteine synthetase. Administration of other precursors of cysteine is feasible. It has long been known that thiazolidine-4-carboxylate is converted to *N*-formylcysteine and to cysteine (29–31), and the 2-methyl derivative of this compound has been reported to be converted to cysteine and acetaldehyde (32). Other compounds that might serve to increase the supply of intracellular cysteine include *N*-acyl, *S*-acyl, and *S*-phosphocysteines, glutathione itself, and cyst(e)ine-containing peptides and esters. In general, it would seem that compounds that are readily transported into cells and that are converted intracellularly by an enzyme-catalyzed mechanism to cysteine would be more effective than those that undergo nonenzymatic conversion to cysteine.

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