Formation of the L-cysteine–glyoxylate adduct is the mechanism by which L-cysteine decreases oxalate production from glycollate in rat hepatocytes

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Formation of thiazolidine-2,4-dicarboxylic acid, the L-cysteine–glyoxylate adduct, is the putative mechanism by which L-cysteine reduces hepatic oxalate production from glycollate [Bais, Rofe and Conyers (1991) *J. Urol.* **145**, 1302–1305]. This was investigated in isolated rat hepatocytes by the simultaneous measurement of both adduct and oxalate formation. Different diastereoisomeric ratios of *cis*- and *trans*-adduct were prepared and characterized to provide both standard material for the enzymic analysis of adduct in hepatocyte supernatants and to investigate the stability and configuration of the adduct under physiological conditions. In the absence of L-cysteine, hepatocytes produced oxalate from 2 mM glycollate at a rate of 822 ± 42 nmol/30 min

per 10^7 cells. The addition of L-cysteine to the incubation medium at 1.0, 2.5 and 5.0 mM lowered oxalate production by 14 ± 2 , 25 ± 3 ($P < 0.05$) and $38 \pm 3\%$ ($P < 0.01$) respectively. These reductions were accompanied by almost stoichiometric increases in the levels of the adduct: 162 ± 6 , 264 ± 27 and 363 ± 30 nmol/30 min per 10^7 cells. Adduct formation is therefore confirmed as the primary mechanism by which L-cysteine decreases oxalate production from glycollate. As urinary oxalate excretion is a prime risk factor in the formation of calcium oxalate stones, any reduction in endogenous oxalate production is of clinical significance in the prevention of this formation.

INTRODUCTION

Urinary oxalate is the most significant risk factor in the formation of calcium oxalate stones, with reductions in urinary oxalate excretion decreasing the risk of stone formation in an exponential manner (Robertson, 1984); thus any reduction in oxalate production produced by treatment with L-cysteine would be of clinical significance. Previous studies from this laboratory indicate that the control of oxalate production is in the final pathway in which the immediate precursors of oxalate, namely glycollate and glyoxylate, are metabolized (Rofe et al., 1976; Bais et al., 1989, 1991a,b). L-Cysteine, a β -aminothiol, is known to react rapidly with glyoxylate under physiological conditions to yield thiazolidine-2,4-dicarboxylic acid, the L-cysteine–glyoxylate adduct. Considerable evidence exists to suggest that adduct formation occurs physiologically (Burns et al., 1984; Hamilton, 1985; Venkatesan and Hamilton, 1986). In addition, orally administered adduct is excreted rapidly and exhibits very low toxicity (LD_{50} for rats and mice = 2 g/kg) (Alary et al., 1989). We have previously investigated the effects of β -aminothiols on oxalate production from glyoxylate *in vitro* (Bais et al., 1991b) and the effect of L-cysteine and L-2-oxothiazolidine-4-carboxylate on urinary oxalate excretion (Baker et al., 1994; Bais et al., 1991b). The present study investigates whether formation of the L-cysteine–glyoxylate adduct is the mechanism by which L-cysteine decreases oxalate production from glycollate in hepatocytes. Glycollate is preferentially used as the oxalate precursor in investigations of the effects of L-cysteine on oxalate production, as it requires transport into the cells before it is metabolized in the peroxisomes by glycollate oxidase to glyoxylate. When glyoxylate is used as the oxalate precursor, adduct formation can occur either inside or outside the cell, whereas any reduction in oxalate production from glycollate caused by L-cysteine can essentially be attributed to the intracellular formation of the adduct.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade and were from the following: glyoxylate from Aldrich (Milwaukee, WI, U.S.A.); oxalate decarboxylase (*Colybia velutipes*) and D-aspartate from Sigma (St. Louis, MO, U.S.A.); L-cysteine from BDH (Poole, Dorset, U.K.); [$1-^{14}C$]glycollate from Amersham International (Amersham, Bucks., U.K.); glycollate from Calbiochem (San Diego, CA, U.S.A.); collagenase H (0.19 unit/mg; *Clostridium histolyticum*) from Boehringer (Mannheim, Germany).

Preparation and characterization of thiazolidine-2,4-dicarboxylic acid, the L-cysteine–glyoxylate adduct

Thiazolidine-2,4-dicarboxylic acid (100% *cis*-adduct) was prepared by mixing equal amounts of aq. 1.6 M (L-cysteine and glyoxylate in a glass scintillation vial (pH not adjusted before addition, final pH 1.4), and the 51% *cis*-adduct was prepared by mixing equal amounts of aq. 1.6 M L-cysteine and glyoxylate in a glass scintillation vial (pH adjusted to 7.4 before addition). After mixing, the vials were evacuated with nitrogen and sealed. The reaction was allowed to proceed for 3 h at 80 °C in a shaking water bath at 80 oscillations/min. At the completion of the reaction the precipitate was collected and washed three times with ethanol before freeze-drying. The precipitate was found to be pure 100% *cis*-adduct or 51% *cis*-adduct respectively. The purity of the preparation and the structure of the adduct were confirmed by capillary tube melting-point determinations, proton n.m.r., KBr disc i.r. spectroscopy and gas chromatography/electron-impact m.s. Proton n.m.r. spectra of the adduct were obtained on a Bruker ACP 300 MHz spectrometer using [3H]dimethyl sulphoxide as the solvent and 1% tetramethylsilane as the reference. Adduct stereochemistry was confirmed by proton n.m.r. (McMillan and Stoodley, 1968). I.r. spectra were obtained

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Figure 1 Proton-n.m.r. spectra (300 MHz) of diastereoisomeric mixtures of thiazolidine-2,4-dicarboxylic acid in $[\text{2H}]$ dimethyl sulphoxide using 1% tetramethylsilane as reference

(a) Fresh 51% *cis*-thiazolidine-2,4-dicarboxylic acid (the insert shows the structure and configuration of *trans*-thiazolidine-2,4-dicarboxylic acid), (b) fresh 100% *cis*-thiazolidine-2,4-dicarboxylic acid (the insert shows the structure and configuration of *cis*-thiazolidine-2,4-dicarboxylic acid) and (c) 72% *cis*-thiazolidine-2,4-dicarboxylic acid resulting from the epimerization of 100% *cis*-thiazolidine-2,4-dicarboxylic acid in $[\text{2H}]$ dimethyl sulphoxide at room temperature over 12 days.

on a Philips SP3 100 spectrophotometer. The 100% *cis*-adduct was trimethylsilylated using *NO*-bis(trimethylsilyl)trifluoroacetamide to ensure volatility for gas chromatographic/mass spectroscopic analysis which was performed on a Hewlett-Packard 5890 gas chromatograph linked to a Hewlett-Packard 5988 quadrupole electron-impact mass spectrometer. $\text{p}K_{\text{a}}$ values were determined by titration of 100% *cis*-adduct with NaOH or HCl while the pH was monitored with a glass electrode.

Isolation of D-aspartate oxidase

Bovine kidney cortex and medulla (580 g) was used to isolate D-aspartate oxidase enzyme purification *b* of Nasu et al. (1982). In step 2, sodium potassium tartrate powder was replaced with DL-malic acid. After step 3, the dialysate was centrifuged at 20000 *g* for 15 min. The resultant supernatant contained 2 mg/ml protein and was used without further purification. Protein concentrations

were determined using Biuret reagent (Bayer Diagnostics, Technicon Dax 48 Autoanalyser).

Enzymic assay of thiazolidine-2,4-dicarboxylic acid

The L-cysteine-glyoxylate adduct was determined spectrophotometrically on a Cobas Bio Analyser using bovine kidney D-aspartate oxidase (Dixon and Kenworthy, 1967). Physiological concentrations of *cis*-adduct are an excellent substrate for D-aspartate oxidase; *trans*-adduct is not a substrate for this enzyme (Hamilton, 1985). In addition, ferricyanide is an efficient electron acceptor. If ferricyanide is used as the terminal electron acceptor for D-aspartate oxidase, the decrease in A_{420} corresponds to half the decrease in substrate concentration (Dixon and Kenworthy, 1967):

As cysteine was shown to rapidly reduce ferricyanide to ferrocyanide causing interference with the assay, residual L-cysteine in the hepatocyte supernatant was complexed with formaldehyde (Ratner and Clarke, 1937). Thus 63.6 μl of 120 mM formaldehyde (final concentration 10 mM) was added to 700 μl of hepatocyte supernatant before boiling for 2 min. The addition of formaldehyde to samples containing adduct did not significantly alter the activity of D-aspartate oxidase in the assay. In addition, the adduct was shown to be stable to boiling. A standard curve was prepared by adding 51% *cis*-adduct to boiled hepatocytes to a final concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 mM total adduct. The standard curve for the adduct assay was linear between 0 and 0.5 mM. The standards and blanks were also treated with formaldehyde before being boiled for 2 min. The 51% *cis*-adduct was used as the standard because L-cysteine and glyoxylate react in the hepatocytes at pH 7.4 to produce this diastereoisomeric ratio giving both standards and samples an equal diastereoisomeric ratio of *cis/trans*-adduct. The assay was started by adding D-aspartate oxidase. The colour reagent contained 376 mM KH_2PO_4 (pH 7.4), 909 mg/l bovine liver catalase (1600 units/mg) and 2.39 mM potassium ferricyanide, and the starting reagent contained 57.7 mM FAD and D-aspartate oxidase suspension at 1.33 g/l protein. FAD and D-aspartate oxidase were mixed at least 15 min before use and incubated at 30 °C to allow recombination of the FAD with the apoenzyme (Dixon and Kenworthy, 1967). The final assay volume was 207 μl made up of 80 μl of sample, 55 μl of colour reagent and 72 μl of starting reagent.

Hepatocyte preparation and incubation conditions

A modification of the original method of Berry and Friend was used to isolate hepatocytes from fed male rats (Porton strain; 300–400 g) by collagenase H perfusion of the livers (Rofe et al., 1976). The isolation medium contained 10 mM Hepes buffer, pH 7.4, 0.05% (w/v) collagenase, 130 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 and 24 mM NaHCO_3 through which carbogen (95% O_2 /5% CO_2) had been bubbled for 10 min. The cells were incubated in isolation medium (without collagenase) containing 1.25 mM anhydrous CaCl_2 (equilibrated with carbogen). Cell viability was 85–95% as assessed by Trypan Blue exclusion. Cells were incubated at a concentration of 10^7 /ml. Incubations were performed in glass scintillation vials flushed with carbogen, capped and incubated for 30 min at 37 °C in a reciprocating shaking water bath at 80 oscillations/min. All incubations were performed in duplicate. The stock solutions of substrate were prepared daily, with the pH being adjusted to 7.4 with either NaOH or HCl.

Figure 2 Electron-impact mass spectrum of the trimethylsilyl derivative of 100% *cis*-thiazolidine-2,4-dicarboxylic acid

The inserts show (a) the structure of the trimethylsilyl derivative of 100% *cis*-thiazolidine-2,4-dicarboxylic acid and the molecular fragment which gives rise to the peak at mass/charge 204 and (b) the molecular ion at mass/charge 321.

Investigations into the effect of L-cysteine on production of [^{14}C]oxalate and $^{14}\text{CO}_2$ from [1- ^{14}C]glycollate by hepatocytes

Hepatocytes were incubated with 2 mM glycollate (0.1 μCi of [1- ^{14}C]glycollate) to determine the ability of L-cysteine to affect production of [^{14}C]oxalate and $^{14}\text{CO}_2$ or 2 mM glycollate to determine the extent of L-cysteine-glyoxylate adduct formation. The incubations were carried out in a final volume of 1.1 ml. L-Cysteine was tested at 1.0, 2.5 and 5.0 mM. To determine [^{14}C]oxalate and $^{14}\text{CO}_2$, incubations were stopped by the addition of 1.5 ml of cold 1 M citric acid, pH 3.0, whereas to determine the L-cysteine-glyoxylate adduct they were stopped by boiling for 2 min.

Assay of $^{14}\text{CO}_2$ and [^{14}C]oxalate

Immediately after the addition of 1.5 ml of cold 1 M citric acid to the cell suspension in the incubation vial, a small inner vial (1.0 $\times$ 4.5 cm) containing 0.2 ml of 1 M hyamine hydroxide in methanol was inserted and the incubation vial capped. After 2 h at room temperature, the inner vial was removed, the exterior washed with distilled water, and the radioactivity, representing metabolic production of $^{14}\text{CO}_2$, measured after the addition of 3.5 ml of scintillant (0.4% 2,5-diphenyloxazole in toluene). Two 1 ml portions of the residual cell suspension were then placed into separate scintillation vials. Then 0.1 ml of oxalate decarboxylase (0.05 unit in 400 mM citric acid, pH 4.5), was added to one vial, an inner hyamine vial (as above for metabolic $^{14}\text{CO}_2$) inserted and the incubation vial capped. To the second vial, only 0.1 ml of 400 mM citric acid buffer, pH 4.5, was added, an inner hyamine vial (as above for metabolic $^{14}\text{CO}_2$) inserted and the incubation vial capped. The latter vial was necessary for the determination of residual $^{14}\text{CO}_2$. The vials were incubated at 37 $^\circ\text{C}$ for 2 h in a reciprocating shaking water bath at 80 oscillations/min. After this time the inner vials were removed, the outsides rinsed and 3.5 ml of scintillant (0.4%, 2,5-diphenyloxazole in toluene) was added before measurement of radioactivity. Internal standards of [U- ^{14}C]oxalate were included in each assay (Rofe et al., 1976). The amount of oxalate produced

was calculated from the difference between the vials with and without oxalate decarboxylase.

Data analysis

All data were examined for statistical significance using the two-sample *t* test on Minitab software. All values are expressed as means $\pm$ S.E.M.

RESULTS AND DISCUSSION

Preparation and characterization of thiazolidine-2,4-dicarboxylic acid

Proton n.m.r. confirmed that thiazolidine-2,4-dicarboxylic acid is the product of the reaction between equimolar amounts of L-cysteine and glyoxylate. At pH 7.4 and pH 1.4, L-cysteine and glyoxylate react to give 51% *cis*-adduct (Figure 1a) and 100% *cis*-adduct (Figure 1b) respectively. Thus initially at pH 7.4 when L-cysteine and glyoxylate react intracellularly they produce the 51% *cis*-adduct. However, slow thermodynamic epimerization of *cis*-adduct to the more stable *trans*-adduct may occur; epimerization of 100% *cis*-adduct in [^2H]dimethyl sulphoxide to the 72% *cis*-adduct, over a 12-day period at room temperature, was confirmed by proton n.m.r. (Figure 1c). Slow thermodynamic epimerization has also been observed with 5,5-dimethyl-thiazolidine-2,4-dicarboxylic acid, the D-penicillamine-glyoxylate adduct (Bringmann et al., 1992). The splitting patterns and chemical shifts observed in the proton-n.m.r. spectra of the adduct agree with predictions based on first principles for the suggested structure (Kemp, 1987). The ratio of calculated mass to observed mass of the molecular ion of the trimethylsilyl derivative of the 100% *cis*-adduct (Figure 2), assuming silylation at both carboxylic acid functional groups, was 1.00, confirming the overall elemental composition of the adduct. In addition, the fragmentation patterns and major ion fragments confirm the overall molecular structure. The i.r. spectra (Figure 3) confirmed the presence of the secondary amine functional group (broad peak at 3600–3200 cm^{-1}) and the carbonyl moieties on the carboxylic acid functional groups (sharp peak at 1680 cm^{-1})

Figure 3 KBr disc I.R. spectra of 100% (—) and 51% (----) *cis*-thiazolidine-2,4-dicarboxylic acid

The sharp peak at 1680 cm^{-1} is indicative of the carbonyl moiety on the carboxylic acid functional groups whereas the broad peak at $3600\text{--}3200\text{ cm}^{-1}$ is indicative of the secondary amine functional group.

(Kemp, 1987). Sharp melting points corresponding to those observed by Forneau et al. (1971) confirmed the purity of both diastereoisomeric preparations: 186°C for 100% *cis*-adduct and 188°C for 51% *cis*-adduct. On reaching the melting point, the samples bubbled vigorously indicating release of CO_2 from the carboxylic acid functional groups. Two pK_a values for 100% *cis*-adduct were found to be 3.90 and 9.25 corresponding to one of

the carboxylic acid functional groups and the secondary amine functional group respectively. It is reasonable to assume that the pK_a for the other carboxylic acid functional group is below 2 as the adduct is a strongly acidic dicarboxylic acid (Aylward and Findlay, 1987). Thus at pH 7.4, the adduct exists with both dicarboxylic acid functional groups deprotonated and the secondary amine functional group protonated.

Investigations into the effect of β -aminothiols on production of [^{14}C]oxalate and $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]glycollate by hepatocytes

Glycollate is metabolized to oxalate in the peroxisomes either directly by glycollate dehydrogenase or indirectly by glycollate oxidase with glyoxylate as an intermediate (Figure 4) (Fry and Richardson, 1979; Yanagawa et al., 1990). Glyoxylate can react readily with L-cysteine to form the L-cysteine-glyoxylate adduct. Adduct formation has previously been postulated as the mechanism by which (a) L-cysteine decreases oxalate production from glyoxylate *in vitro* (Bais et al., 1991b) and (b) L-cysteine and L-2-oxothiazolidine-4-carboxylate lower urinary oxalate excretion (Baker et al., 1994). In the present study hepatocytes incubated in the absence of L-cysteine produced oxalate from 2 mM glycollate at a rate of $822 \pm 42\text{ nmol/30 min}$ per 10^7 cells and CO_2 at a rate of $63 \pm 3\text{ nmol/30 min}$ per 10^7 cells. However, incubation in the presence of 1.0, 2.5 and 5.0 mM L-cysteine decreased oxalate production from glycollate (Figure 5) (114 ± 15 , 201 ± 24 ($P < 0.05$) and 315 ± 27 ($P < 0.01$) nmol/30 min per 10^7 cells respectively) whereas CO_2 production was not significantly affected by altering the concentration of L-cysteine. The reduction in oxalate production observed with 1.0, 2.5 and 5.0 mM L-cysteine was accompanied by an increase in the levels of the L-cysteine-glyoxylate adduct (Figure 5): 162 ± 6 ($P < 0.001$),

Figure 4 Proposed model for the generation of glyoxylate, the L-cysteine-glyoxylate adduct, CO_2 and oxalate from glycollate and L-cysteine in isolated rat hepatocytes

Figure 5 Effect of 1.0, 2.5 and 5.0 mM L-cysteine on oxalate production (■), CO₂ production (●) and thiazolidine-2,4-dicarboxylic acid formation (▲) from 2 mM glycolate in isolated rat hepatocytes

Incubation in the absence of L-cysteine produced oxalate from 2 mM glycolate at a rate of 822 ± 42 nmol/30 min per 10^7 cells and CO₂ at a rate of 63 ± 3 nmol/30 min per 10^7 cells. Results are expressed as means $\pm$ S.E.M. for three independent hepatocyte preparations. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with controls.

264 ± 27 ($P < 0.01$) and 363 ± 30 ($P < 0.01$) nmol/30 min per 10^7 cells respectively. A significant correlation between the decrease in oxalate production and the formation of the L-cysteine-glyoxylate adduct was shown to exist: adduct formation (nmol/30 min per 10^7 cells) = $(0.84 \pm 0.17) + (1.09 \pm 0.03)$ (decrease in oxalate) ($r = 0.94$; $P < 0.001$). This observation supports the view that formation of L-cysteine-glyoxylate adduct is the mechanism by which L-cysteine decreases oxalate production from glycolate.

In addition to oxalate production, glyoxylate can be converted into glycine by alanine-glyoxylate aminotransferase. Rat liver cytosol, microsomes, lysosomes and peroxisomes contain 25% of the total cellular activity of this enzyme (Rowell et al., 1972), and thus the observation that adduct formation accounted for $9 \pm 3\%$ more glyoxylate than the decrease in oxalate can be explained by the fact that the additional adduct comes from glyoxylate that under normal conditions would undergo transamination (Figure 4).

CO₂ produced in the metabolism of glycolate results from decarboxylation of glyoxylate, the major portion of which occurs in the mitochondria catalysed by α -oxoglutarate-glyoxylate carboxylase (O'Fallon and Brosemer, 1977) with a smaller amount of H₂O₂-dependent non-enzymic decarboxylation occurring in the peroxisomes (Rofe et al., 1976) (Figure 4). Incubation of hepatocytes with 2 mM glycolate produced 13.0 ± 0.9 -fold more oxalate than CO₂. This observation suggests that oxidation of glycolate via glycolate oxidase produced glyoxylate that cannot easily be decarboxylated via mitochondrial α -oxoglutarate-glyoxylate carboxylase, an observation that can be attributed to compartmentalization. Glyoxylate produced by the oxidation of glycolate in the peroxisomes can be oxidized or reduced to oxalate or glycolate by lactate dehydrogenase in the cytosol (Figure 4) before reaching the mitochondria for decarboxylation. Thus the majority of CO₂ produced from glycolate is probably

from the H₂O₂-dependent non-enzymic decarboxylation of glyoxylate in the peroxisomes. The observation that L-cysteine had no effect on CO₂ production while significant diminishing oxalate production through formation of the L-cysteine-glyoxylate adduct suggests that the majority of CO₂ production in the peroxisome occurs before L-cysteine-glyoxylate adduct formation. H₂O₂ and glyoxylate are formed as a result of the oxidation of glycolate by glycolate oxidase (Schuman and Massey, 1971). Thus, with respect to the rest of the cell, glyoxylate and H₂O₂ are present in relatively high concentrations near the active site of this enzyme. It can be postulated that, as glyoxylate and H₂O₂ diffuse from the enzyme surface, the glyoxylate is rapidly decarboxylated by the high local concentration of H₂O₂, resulting in CO₂ production. This process will slow as diffusion from the enzyme surface proceeds and the relative concentrations of glyoxylate and H₂O₂ are diminished. In addition, the high activity of catalase in peroxisomes will deactivate the H₂O₂ as it diffuses from the enzyme surface. The remaining glyoxylate diffusing out can then undergo L-cysteine-glyoxylate adduct formation (Figure 4).

The results of this study indicate that other β -aminothiols, particularly D-cysteine and D-penicillamine, may be able to decrease oxalate production from glycolate through formation of the respective β -aminothiol-glyoxylate adduct. In addition, it now seems probable that the reduction in urinary oxalate excretion previously observed on feeding L-cysteine (Bais et al., 1991b) and L-2-oxothiazolidine-4-carboxylate (Baker et al., 1994) is due to the formation of the L-cysteine-glyoxylate adduct.

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