INDUCTION OF CYSTEINE DIOXYGENASE ACTIVITY BY ORAL ADMINISTRATION OF CYSTEINE ANALOGUES TO THE RAT: IMPLICATIONS FOR DRUG EFFICACY AND SAFETY

E.S. Roopnarinesingh[1], G.B. Steventon[2], R.M. Harris[3], R.H. Waring[3] and S.C. Mitchell[1]*

[1]*Section of Biological Chemistry, Division of Biomedical Sciences, Faculty of Medicine, Imperial College London,*
[2]*Department of Pharmacy, School of Health and Life Sciences, King's College London, London, and*
[3]*School of Biosciences, University of Birmingham, Birmingham, UK*

SUMMARY

One of the major steps in the oxidation of the sulphur-containing amino acid, L-cysteine, is the production of cysteine sulphinic acid, catalysed by the enzyme cysteine dioxygenase. This enzyme plays a key role in the intermediary metabolism of sulphur-containing compounds. The activity of this crucial enzyme is known to be influenced by sulphur-compound intake, being increased in animals fed an excess of L-cysteine or methionine. However, the affects on this enzyme of the chronic administration of drugs similar in structure to cysteine are unknown. This has now been investigated using the anti-rheumatic agent, D-penicillamine, and the mucoactive compound, *S*-carboxy-

* Author for correspondence:
Dr. S.C. Mitchell
Section of Biological Chemistry
Division of Biomedical Sciences
Faculty of Medicine
Imperial College London
Sir Alexander Fleming Building
South Kensington
London SW7 2AZ, UK
e-mail: s.c.mitchell@imperial.ac.uk

methyl-L-cysteine. Repeated oral administration of these sulphur-containing drugs to male Wistar rats for five consecutive days led to a significant increase in hepatic cysteine dioxygenase activity. This increase in the production rate of cysteine sulphinic acid remained evident until returning to control levels four days after cessation of drug administration. These observations provide evidence that these two drugs interact with the intermediary biochemistry of sulphur compounds and may provide hitherto unappreciated insights into mechanisms by which therapeutic effects and adverse reactions may occur.

KEY WORDS

D-penicillamine, *S*-carboxymethyl-L-cysteine, L-cysteine, sulphinic acid, *S*-oxidation, rat

INTRODUCTION

Chemicals with a structural similarity to the sulphur-containing amino acid cysteine have been employed for many years as therapeutic agents, within both veterinary practice and human medicine. One such compound is D-penicillamine, an anti-rheumatic drug, which is also used for the management of hepatolenticular degeneration (Wilson's disease) and cystinuria as well as certain metal ion poisonings. *S*-Carboxymethyl-L-cysteine, a mucoactive agent, is another derivative, employed as an expectorant in asthma, bronchitis and cystic fibrosis, and also as an adjunct in the treatment of suppurative otitis media (glue ear) and chronic obstructive airway disease (COPD; airflow restriction) (Fig. 1).

During their passage through the mammalian body the sulphur moieties contained within these two drugs are known to be cleaved and oxidised, in part, to yield inorganic sulphate, and it has been suggested that these compounds may interfere in some way with the complex intermediary biochemistry of cysteine metabolism /1-4/. Indeed, it has recently been shown that both of these compounds, when co-administered with [^{35}S]-L-cysteine, were able to decrease the subsequent urinary excretion of metabolically derived radiolabelled inorganic sulphate /5/.

$HSCH_2CH(NH_2)COOH$	$HOOCCH_2SCH_2CH(NH_2)COOH$	$HSC(CH_3)_2CH(NH_2)COOH$
L-Cysteine	S-Carboxymethyl-L-cysteine	D-Penicillamine

Fig. 1: Drugs used in this study and their structural comparison to cysteine.

One of the major steps in the oxidation of the sulphur-containing amino acid, L-cysteine, is the production of its sulphinic acid, catalysed by the enzyme cysteine dioxygenase. From this pivotal point, cysteine sulphinic acid is further metabolised via other key intermediates, including hypotaurine, β-sulphinylpyruvate and cysteic acid, thereby exerting a major controlling influence over sulphur-compound catabolism and the eventual production of inorganic sulphate. The activity of this enzyme is known to be affected by a variety of factors including the administration of excess L-cysteine and methionine within the diet /6/. It is possible that other sulphur-containing compounds similar to cysteine in structure, and chronically administered as drugs, may also affect the activity of cysteine dioxygenase. This has now been investigated in the present study following the repeated oral administration of D-penicillamine and *S*-carboxymethyl-L-cysteine to rats.

MATERIALS AND METHODS

Chemicals

Ammonium iron(II) sulphate hexahydrate, bovine serum albumin, *S*-carboxymethyl-L-cysteine (carbocisteine; 3-[(carboxymethyl)thio] alanine), Coomassie brilliant blue G-250 dye (acid blue 90), L-cysteic acid monohydrate (3-sulpho-L-alanine), L-cysteine hydrochloride (2-amino-3-mercaptopropanoic acid hydrochloride), L-cysteine sulphinic acid monohydrate (3-sulphino-L-alanine), hydroxylamine hydrochloride, nicotinamide adenine dinucleotide (β-NAD^+; DPN) and D-penicillamine (3-mercaptovaline) were supplied by Sigma-Aldrich Chemical Co. (Gillingham, Kent, UK). Corn oil was purchased from a local store. Other chemicals were of analytical grade, with solvents being of h.p.l.c. quality, and all were readily available within the laboratory. Radiolabelled [^{35}S]-L-cysteine hydrochloride (sp. act. 103 mCi/mol, purity >99%) was obtained from Amersham International plc. (Amersham, Buckinghamshire, UK).

Animal investigations

Thirty-six male Wistar rats (200 ± 12 g; Harlan, Oxford Laboratory Animal Centre, Bicester, UK) were acclimatized in a 12-hour

night/day cycled laboratory (at 24°C) for at least one week. These animals were maintained on a standard pelleted diet (Labsure CRM Pellets; K & K Greef Ltd, Croydon, UK) with free access to water. After this time the rats were randomly assigned to three groups of twelve, with one group acting as control animals. Rats within this control group were given a dose of corn oil (2.5 ml/kg body weight) via gastric intubation at 09.30 h whereas those within the two test groups received the compound under investigation (either D-penicillamine or *S*-carboxymethyl-L-cysteine) as a suspension in corn oil (6.35 mmol/2.5 ml/kg body weight). This dose of test compound was about 10-fold the daily dietary intake of L-cysteine (*c.* 22 mg). The animals were kept in separate metabolism cages and were weighed each morning with the above dosing procedure being repeated at 09.30 h for five consecutive days.

Measurement of cysteine dioxygenase activity

On the fifth day, one hour after the administration of the final dose, four rats from each test group were euthanized by cervical dislocation and their livers removed by dissection. This procedure was repeated with another four rats from each test group on the sixth day with the remainder being euthanized on the ninth day. Following the removal of excess fat, the livers were weighed and chopped in ice-cold buffer (50 mM potassium phosphate, pH 6.8; 25% w/v) before being homogenised (Potter Elvejhem homogeniser; B. Braun A.G., Melsungen, Germany). An initial centrifugation (10,000 *g*, 30 min, 4°C) of the homogenate removed cell debris prior to further centrifugation (100,000 *g*, 60 min, 4°C) of the resulting supernatant to separate and provide the required cytosolic fraction.

The procedure employed was that described previously with minor modifications /7/. Incubations were undertaken in a final volume of 2 ml consisting of 50 mM potassium phosphate buffer (pH 6.8), 0.25 mM ammonium iron(II) sulphate hexahydrate, 2 mM NAD^+, 10 mM hydroxylamine hydrochloride, 0-12 mM L-[^{35}S]-cysteine hydrochloride (as substrate) and 1.5 ml cytosol (enzyme preparation). Solutions of hydroxylamine hydrochloride and L-[^{35}S]-cysteine hydrochloride were neutralised to pH 6.8 with NaOH before use. Tubes were pre-incubated at 37°C for 5 min before initiation of the reaction by addition of substrate (L-[^{35}S]-cysteine hydrochloride) followed by incubation for a further 3 min. The reaction was terminated by the

addition of trichloroacetic acid (0.5 ml, 5% w/v) and barium chloride (0.5 ml; saturated solution). Zero-time incubations were used as control blanks. All investigations were undertaken in duplicate. The reaction tubes were vortex mixed and centrifuged (2,000 *g*; 10 min) to remove the supernatant.

Quantification of reaction products

The reaction products were isolated and quantified by slight modification of a previously detailed procedure /7-9/. Briefly, aliquots (1 ml) of the incubation supernatant were applied to cation exchange columns (0.5 x 5.0 cm; Dowex 50WX8-400 [200-400 mesh] H^+ form; Sigma-Aldrich Chemical Co.), previously equilibrated to pH 2 with 10 mM HCl, and eluted with an acid wash (7 ml; 10 mM HCl). At pH 2 these columns retain cysteine and other amino acids but permit the passage of cysteine sulphinic acid and cysteic acid, the latter being formed via non-enzymatic oxidation of cysteine sulphinic acid /9,10/. Thin layer chromatography (cellulose plate 0.2 mm; Merck, Darmstadt, Germany; solvent system: n-butanol: glacial acetic acid: water, 3:1:1 by vol.) and localisation via radiometric analysis confirmed the presence of cysteine sulphinic acid (Rf 0.12) and cysteic acid (Rf 0.07) [cysteine Rf 0.32] as the only radioactive components present in the column eluent.

Measurement of radioactivity

Aliquots of the Dowex column eluent (1.0 ml) were added directly to scintillation fluid (3 ml; 'Ecoscint'; National Diagnostics, Manville, NJ, USA) and counted by liquid scintillation spectrometry using a Packard Tri-Carb 4640 scintillation counter (Canberra-Packard Instruments, Paingbourne, Berkshire, UK) with internal standards being used for quench correction.

Measurement of protein concentration

Protein concentrations were estimated using a protein-dye (Coomassie brilliant blue) binding reaction previously described in detail /11/. Bovine serum albumin at appropriate dilution was employed to construct a standard curve.

RESULTS AND DISCUSSION

The rate of enzyme-catalysed oxidation of L-cysteine to cysteine sulphinic acid was determined separately for each liver employing a range of initial L-cysteine substrate concentrations (0-12 mM). Within the control group of animals no significant differences were observed, for a given substrate concentration, between the reaction rates determined on days 5, 6 and 9. However, when compared to these control values, it could be seen that the reaction rates measured following chronic oral administration of D-penicillamine and *S*-carboxymethyl-L-cysteine were significantly greater on days 5 and 6, with this being most evident at the higher concentrations of L-cysteine ($p < 5\%$; Student's *t*-test) (Fig. 2). Although these reaction rate enhancements were not dramatic, they were readily demonstrable, and usually elevated to the region of 150% of the corresponding control values (D-penicillamine: day 5, 164 ± 22%, day 6, 129 ± 12%; *S*-carboxymethyl-L-cysteine: day 5, 157 ± 15%, day 6 155 ± 9%; mean ± S.D.). When measured on day 9, four days after the cessation of oral drug administration, these reaction rates had decreased and were not significantly different from the corresponding control values (Fig. 2, Table 1).

An increase in enzyme activity was evident following chronic dosing with either D-penicillamine or *S*-carboxymethyl-L-cysteine daily for five consecutive days. Previous studies have shown that such a short dosing regimen employing increased concentrations of the natural substrate, L-cysteine, was sufficient to measurably affect cysteine dioxygenase activity /12,13/. Such enzyme induction, in response to repeated or chronic exposure to a xenobiotic, usually requires new enzyme synthesis and achieves this by affecting genetic material. However, activation by post-translational mechanisms may occur, such as mobilization of pre-existing enzyme, as has been suggested previously for cysteine dioxygenase /14/. Once dosing of the xenobiotic had ceased, the measured enzyme activity returned to normal (control) values within 4 days with indications of this trend being evident after 24 h.

After the cessation of dosing a certain proportion of the compounds would have remained within the animal body awaiting excretion. In the rat, the oral uptake of D-penicillamine has been reported to lie between 40% and 70% with over 50% of this amount being eliminated

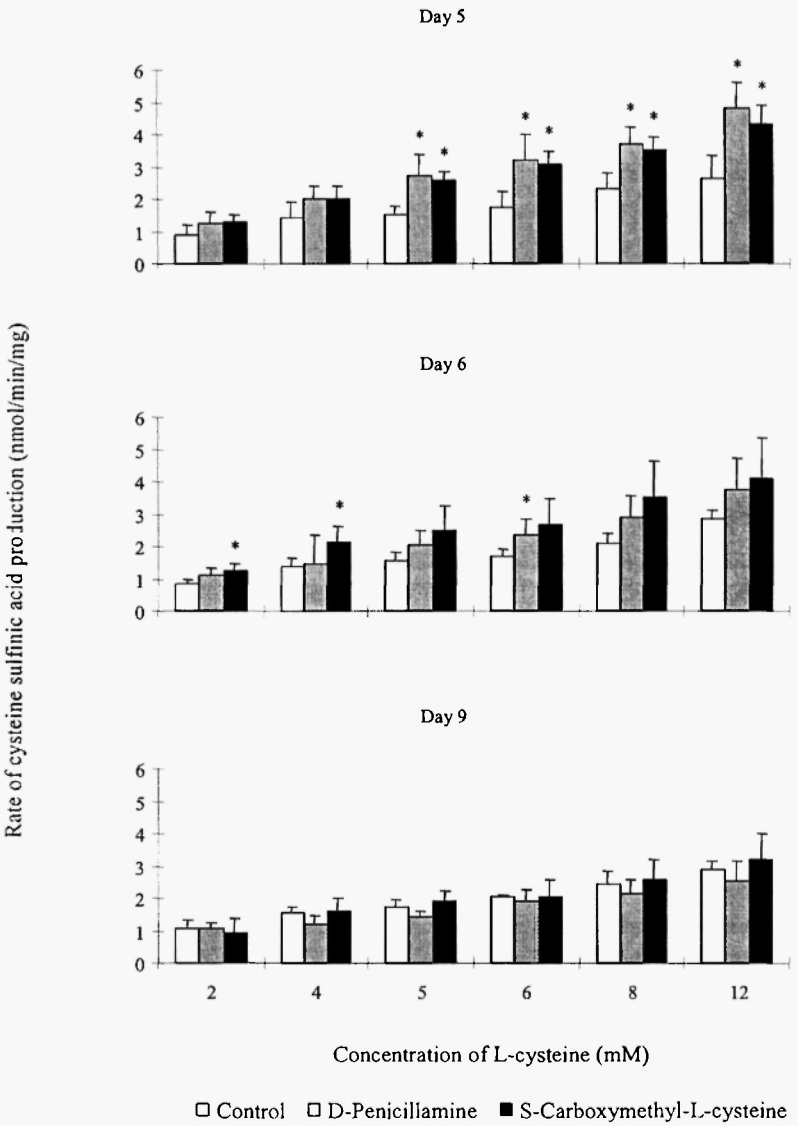

Fig. 2: The effects of five days chronic administration of D-penicillamine and *S*-carboxymethyl-L-cysteine on [^{35}S]-cysteine sulphinic acid production from [^{35}S]-L-cysteine by rat hepatic cysteine dioxygenase activity. Day 5 values were obtained 1 h after the final drug dose, day 6 after 25 h and day 9 after 97 h. Values are expressed as means ± S.D. (n = 4). * = values significantly differed from corresponding control values ($p < 5\%$; Student's *t*-test with Bessel's correction).

TABLE 1

Rate of L-cysteine oxidation in rat liver cytosol after daily oral pretreatment with D-penicillamine or *S*-carboxymethyl-L-cysteine for five consecutive days

L-Cysteine conc. (mM)	Rate of cysteine sulphinic acid production (nmol/min/mg)								
	Controls			D-Penicillamine			*S*-Carboxymethyl-L-cysteine		
	day 5	day 6	day 9	day 5	day 6	day 9	day 5	day 6	day 9
2	0.90±0.28	0.86±0.11	1.05±0.29	1.23±0.36	1.10±0.25	1.06±0.18	1.29±0.21	1.25±0.20†	0.92±0.45
4	1.44±0.48	1.37±0.27	1.57±0.15	2.02±0.40	1.45±0.92	1.18±0.30	2.00±0.38	2.14±0.50†	1.58±0.43
5	1.52±0.28	1.56±0.25	1.75±0.21	2.73±0.66†	2.03±0.47	1.42±0.19	2.57±0.28†	2.48±0.78	1.89±0.34
6	1.73±0.48	1.69±0.24	2.03±0.08	3.21±0.78†	2.37±0.48†	1.93±0.32	3.06±0.42†	2.68±0.78	2.05±0.54
8	2.33±0.46	2.10±0.31	2.46±0.37	3.68±0.55†	2.88±0.68	2.15±0.43	3.50±0.42†	3.50±1.14	2.57±0.64
12	2.62±0.71	2.83±0.30	2.87±0.27	4.80±0.78†	3.74±0.98	2.52±0.62	4.29±0.59†	4.07±1.27	3.18±0.83

† = values significantly different from corresponding control values ($p < 5\%$; Student's *t*-test with Bessel's correction).

via the kidneys within 0-48 h /15-18/. *S*-Carboxymethyl-L-cysteine is also known to appear rapidly in tissues following gastrointestinal administration from where it almost totally absorbed /19,20/ with over 55% of the dose being excreted in 0-24 h urine /11,21/. Using this information, a rough estimate of the amount of D-penicillamine (day 5, 200-350 mg; day 6, 130-230 mg; day 9, 40-70 mg) and *S*-carboxymethyl-L-cysteine (day 5, 370-410 mg; day 6, 150-170 mg; day 9, 5-15 mg) derived material remaining within the rats following cessation of dosing may be made. Previous work has demonstrated that co-administration of D-penicillamine or *S*-carboxymethyl-L-cysteine with [^{35}S]-L-cysteine decreased the subsequent production and/or excretion of radiolabelled sulphate formed via the oxidation of the cysteine sulphur moiety /5/. If these xenobiotics were present within the liver incubates a decrease in rate would have been predicted if the compounds affected (acted as competitive inhibitors) the cysteine dioxygenase catalysed step in the oxidation of cysteine sulphur to sulphate. This was not observed; perhaps the effective concentrations were too low.

Most medications are prescribed for chronic administration and these repeat doses undoubtedly have an effect at many points within intermediary metabolism resulting in mainly unknown subclinical alterations in homeostasis. With respect to possible enzyme induction it is usually only the more apparent enzymes, such as cytochrome(s) P450, which are investigated. The present data indicate that other less obvious enzymes may also be affected with unappreciated consequences. The overall outcome of the influence of D-penicillamine and *S*-carboxymethyl-L-cysteine on the oxidation of L-cysteine via cysteine dioxygenase within the extremely complex intermediary biochemistry of sulphur-containing compounds is unclear. Metabolic switching, variations in flux through several interrelated pathways and alterations in sulphate availability are just a few of the possibilities. Such perturbations also may provide new insights into the underlying mechanisms by which these drugs bring about their therapeutic effects.

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