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CYSTEINE *ISO*PROPYLESTER PROTECTS AGAINST PARACETAMOL-INDUCED TOXICITY

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Abstract—Cysteine *iso*propylester (CIPE), a novel ester of cysteine, has been synthesized in order to evaluate its potential as a chemoprotectant. The increased lipophilicity of the ester relative to cysteine should facilitate its entry into cells where, following hydrolysis, it should act as an intracellular source of cysteine or be utilized for the synthesis of glutathione so protecting the cell against various types of chemical insult. In this study, we evaluate the ability of CIPE to protect against paracetamol-induced hepatotoxicity in mice. When administered to mice, CIPE produced a rapid but transient elevation of levels of non-protein sulphydryls (NPSH) in liver, lung, kidney and spleen. The greatest increase in NPSH was seen in the lung, but after 60 min all NPSH values had returned to control levels, demonstrating the capacity of the mouse to rapidly metabolize both CIPE and cysteine. In mice pretreated with benzo(*a*)pyrene, CIPE protected against paracetamol-induced toxicity as measured by the prevention of mortality, the fall in hepatic NPSH and the decreased elevation of serum transaminases. CIPE (1.5 mmol/kg) appeared as effective as *N*-acetylcysteine (1.5 mmol/kg). Higher doses of CIPE (3.0 mmol/kg) alone were toxic to mice induced with benzo(*a*)pyrene but not to control or phenobarbitone-induced mice. The mechanism of this increased toxicity is unclear. CIPE has a short *in vivo* half life but was capable of protecting against paracetamol-induced toxicity. The potential of CIPE and other related cysteine esters to act as chemoprotectants merits further investigation.

Glutathione (GSH‖) is widely distributed amongst a variety of cell types, where it is involved in the maintenance of cellular homeostasis [1]. Several roles for GSH have been proposed including cellular defence [1], modulation of enzymic activities [2], as a reservoir for the amino acid L-cysteine [3] and in leukotriene biosynthesis [4]. Particular attention has been paid to the various roles of GSH in cellular defence against toxic insults. Intracellular GSH may react with electrophiles either directly or in reactions catalysed by GSH-*S*-transferase [5]. GSH is also utilized by GSH peroxidase to protect against H_2O_2, organic hydroperoxides and lipid hydroperoxides [6]. Cellular GSH status is also a major determinant of susceptibility to radiation [7].

Modulation of tissue GSH levels may greatly influence the susceptibility of various organs to different chemicals [8]. For example, prior depletion of hepatic GSH with diethylmaleate potentiates the toxicity of paracetamol to mice [9]. In order to evaluate the potential of GSH to protect against various toxic insults, a variety of different methods have been utilized to elevate intracellular GSH. Exogenous GSH is unable to cross cell membranes as the intact tripeptide [10], although isolated type II alveolar epithelial cells are able to accumulate GSH without prior extracellular degradation [11]. Liposomally entrapped GSH was much more effective in protecting against paracetamol toxicity than exogenous GSH [12]. In order to facilitate the membrane transport of GSH, a number of hydrophobic esters of GSH have been synthesized which following hydrolysis elevate intracellular GSH [13]. GSH monoethylester enhances intracellular GSH in cultured endothelial cells and protects against H_2O_2-induced injury [14]. GSH isopropylester elevates GSH in several mouse organs [15] and prevents the skeletal muscle degeneration associated with sustained GSH depletion following prolonged exposure to DL-buthionine-*SR*-sulphoximine [16]. GSH isopropylester also protects against the toxicity of mercuric chloride and the anti-cancer drug cisplatin [17, 18], and both the methyl and the isopropyl esters of glutathione protect against the hepatotoxicity of paracetamol [15, 19].

A key factor in the maintenance of intracellular GSH is the supply of L-cysteine [1, 3]. Unlike GSH, cysteine is transported into several cell types using neutral amino acid transport systems [20]. Following enzymic hydrolysis by 5-oxoprolinase, OTZ acts as a cysteine delivery system and enhances intracellular cysteine to promote GSH synthesis. Initial reports suggested that OTZ protects mice against paracetamol toxicity [21, 22] but it may not be as effective as initially proposed [15]. In isolated hepatocytes, GSH synthesis can also be stimulated by the amino acid L-methionine, a

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‖ Abbreviations: CIPE, cysteine *iso*propylester; NPSH, non-protein sulphydryls; GSH, glutathione; OTZ, L-2-oxothiazolidine-4-carboxylate; SGOT, serum glutamate-oxaloacetate; SGPT, glutamate-pyruvate transaminase.

cysteine precursor, protecting against bromobenzene toxicity [23].

In this study, we consider a novel cysteine delivery system using cysteine esters to increase intracellular cysteine after enzymic cleavage by esterases. We suggest that administration of cysteine esters may alter the overall thiol status of an organ in two ways. Firstly, the provision of excess cysteine, a substrate for γ-glutamylcysteine synthetase, will promote synthesis of intracellular GSH. Secondly, cysteine may itself be protective as it is known to react with reactive electrophiles more rapidly than glutathione [24].

Several cysteine esters have been synthesized and CIPE has been shown to protect against the acute pulmonary oedema induced by perfluoroisobutylene, a pyrolysis product of polytetrafluoroethylene (Teflon) [25]. In this study we investigate the potential of CIPE to protect against the hepatotoxicity of paracetamol. At normal doses, paracetamol is metabolized primarily to its glucuronic acid and sulphate ester conjugates, whereas at much higher doses sulphation is saturated and more of the paracetamol is metabolized by cytochrome P450 to *N*-acetyl-*p*-benzoquinonimine which normally reacts with GSH. However after overdosage, intracellular GSH becomes depleted and *N*-acetyl-*p*-benzoquinonimine may induce toxicity either by binding covalently to cellular macromolecules [26] or by causing an oxidative stress [27]. In these studies CIPE was shown to be rapidly metabolized by mice resulting in a transient elevation in NPSH in lung, liver, kidney and spleen. CIPE was as effective as *N*-acetylcysteine in protecting against paracetamol-induced toxicity.

MATERIALS AND METHODS

Materials. Cysteine isopropyl ester hydrochloride was synthesized as described by Lailey *et al.* [25]. All other chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.) or BDH Laboratory Supplies (Poole, U.K.).

Animals. Male BALBc mice (17–20 g) were obtained from Bantin and Kingman (Hull, U.K.). Animals were housed under a 12 hr light/dark cycle and allowed free access to food and water during and for at least 7 days prior to experiments. CIPE and other sulphydryl compounds were administered i.p. immediately after dissolving in 0.9% saline. To induce cytochrome P450 activity, mice were injected i.p. with either benzo(*a*)pyrene (20 mg/kg, in sesame oil) or phenobarbitone (80 mg/kg, in 0.9% saline) for 3 consecutive days. Paracetamol (400 mg/kg) was dissolved in propylene glycol and injected i.p. at the same time as CIPE. All compounds were given at a dose volume of 5 mL/kg.

Procedure. To study the short term effects of CIPE on mouse organ thiol (-SH) status, mice were killed by cervical dislocation 5, 15, 30 and 60 min after dosing with CIPE. A portion of unperfused organ (liver, lung, kidney and spleen) was homogenized and assessed for NPSH by the method of Ellman [28]. This method is capable of detecting all NPSH including glutathione, cysteine and CIPE.

In order to estimate the ability of CIPE to prevent paracetamol toxicity, hepatic NPSH was monitored as outlined above at specified time intervals up to 24 hr. Blood samples were also taken by cardiac puncture into heparinized tubes and analysed colourimetrically for SGOT and SGPT activity, after derivatization with 2,4-dinitrophenylhydrazine [29], as markers of liver damage.

Assessment of CIPE or *N*-acetylcysteine as protectants against paracetamol from 24–168 hr was made using mortalities and body weight changes as indicators of toxicity. The toxicities of CIPE and *N*-acetylcysteine were assessed in control mice or in mice induced with benzo(*a*)pyrene or phenobarbitone.

Statistical analysis. The results were analysed using an unpaired *t*-test. Data are expressed as mean values ± SEM; $P < 0.05$ was considered to be significant.

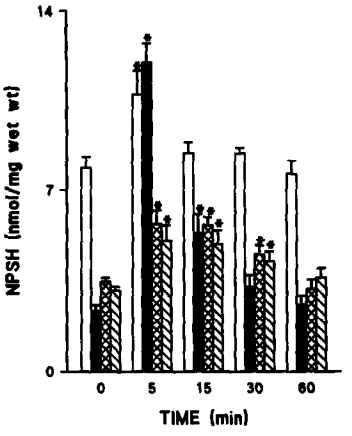

Fig. 1. Elevation of tissue NPSH by CIPE. Mice were dosed with CIPE (3.0 mmol/kg, i.p.) and killed at the indicated times. Homogenates of liver (□), lung (■), kidney (⊠) and spleen (▨) were prepared and NPSH levels measured by the method of Ellman [28]. Each bar represents the mean (±SEM) from results obtained with not less than six animals, assessed on separate occasions. Each NPSH value from CIPE-treated mice was compared with its corresponding zero time control; * $P < 0.05$.

RESULTS

Effect of CIPE on NPSH levels

CIPE produced a transient elevation of NPSH levels in liver, lung, kidney and spleen (Fig. 1). Maximum increases were observed 5 min after administration when the increase in the lung (521% of control) was particularly striking (Fig. 1). These increases were very transient, and pulmonary and hepatic NPSH values fell rapidly and approached control values after 30 min. The elevated NPSH levels noted in the spleen and kidney, while not as pronounced as rises in pulmonary NPSH, followed a similar pattern, reaching control levels between 30 and 60 min.

Effect of CIPE on paracetamol-induced changes in hepatic NPSH and serum enzymes

As the elevation in NPSH following administration

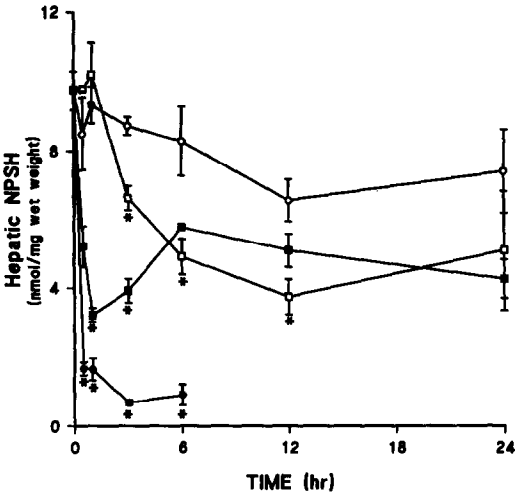

Fig. 2. Effect of CIPE on hepatic NPSH values in mice exposed to paracetamol. Mice were induced with benzo(*a*)pyrene (20 mg/kg, i.p.) for 3 consecutive days. Control mice (○) or mice dosed with paracetamol (400 mg/kg, i.p.) (●) or CIPE (3.0 mmol/kg, i.p.) (□) either alone or in combination (■) with paracetamol were killed at the indicated times. Mice treated with paracetamol alone died within the first 12 hr of exposure. Each point represents the mean (±SEM) from three animals, assessed on separate occasions. NPSH values were compared to their corresponding controls at each time point; * $P < 0.05$. NPSH levels in mice exposed to both CIPE and paracetamol were significantly different from those in mice treated with paracetamol alone.

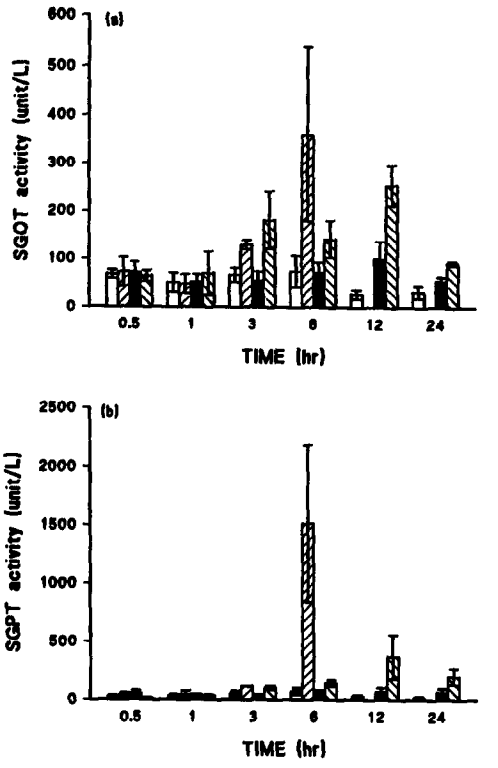

Fig. 3. Effect of CIPE on SGOT (a) and SGPT (b) activities in mice exposed to paracetamol. Mice were dosed with benzo(*a*)pyrene (20 mg/kg, i.p.) for 3 consecutive days. Control mice (□) or mice dosed with paracetamol (400 mg/kg, i.p.) (▨) or CIPE (3.0 mmol/kg, i.p.) (■) either alone or in combination with paracetamol (▧) were killed at the indicated times. Blood samples were drawn by cardiac puncture at the indicated time and assessed for serum transaminase levels. All mice treated with paracetamol alone died within the first 12 hr of exposure. Each point represents the mean (±SEM) of three animals, assessed on separate occasions.

of CIPE was only very transient, we rationalized that any protection afforded directly by the cysteine *per se* may only be of short duration. We therefore required a model of paracetamol toxicity which resulted in a rapid decline of hepatic GSH to coincide with the extra thiol (-SH) groups available due either to CIPE or its hydrolysed product cysteine. In order to accomplish this, mice were pretreated with benzo(*a*)pyrene to induce cytochrome P450 which following paracetamol administration (400 mg/kg) resulted in a rapid and sustained fall in NPSH (>80%) within 30 min (Fig. 2). In contrast when mice were induced with phenobarbitone, paracetamol failed to produce such a rapid and sustained fall in hepatic NPSH (data not shown), so all subsequent experiments unless otherwise stated were carried out with mice pretreated with benzo(*a*)pyrene.

Co-administration of CIPE together with paracetamol partially prevented the extensive decrease in hepatic NPSH observed with paracetamol alone (Fig. 2). After 1 hr, a 65% decrease in NPSH was observed compared to an 82% fall in mice treated solely with paracetamol and after 6 hr a restoration to 75% of the control value was noted. A diurnal variation in hepatic GSH was observed in control mice (Fig. 2) in agreement with previous findings [30, 31]. CIPE (3.0 mmol/kg) caused an initial but short-lived elevation in NPSH followed by a prolonged decrease such that restoration to control values was not observed, even at 24 hr (Fig. 2).

Paracetamol (400 mg/kg) produced a very marked elevation in both SGOT and SGPT which was maximal 6 hr after treatment (Fig. 3). CIPE (3.0 mmol/kg) alone had no effect on either of the transaminases but it inhibited the elevation in SGOT and SGPT caused by paracetamol (Fig. 3). The protection afforded by CIPE against paracetamol-induced loss of NPSH and increases in SGOT and SGPT suggested that CIPE may also protect against paracetamol-induced lethality.

Effect of CIPE on paracetamol-induced mortality

In mice pretreated with benzo(*a*)pyrene, paracetamol (400 mg/kg) caused a 62% mortality within 24 hr. CIPE (3.0 mmol/kg) completely prevented the paracetamol-induced lethality up to 24 hr but little or no protection was evident at later times (Table 1). However, surprisingly CIPE (3.0 mmol/kg) alone was observed to be toxic and after 48 hr fatalities were noted. These results were in marked

Table 1. Effect of CIPE and *N*-acetylcysteine on paracetamol-induced mortality

Treatment	% Mice surviving Days after exposure to paracetamol and/or thiol							
	0	1	2	3	4	5	6	7
Control	100	100	100	100	100	100	100	100
Paracetamol (400 mg/kg)	100	38	24	24	19	19	19	19
CIPE (3.0 mmol/kg)	100	100	100	78	67	67	56	56
Paracetamol + CIPE (3.0 mmol/kg)	100	83	50	33	33	33	33	33
CIPE (1.5 mmol/kg)	100	100	100	100	100	100	100	100
Paracetamol + CIPE (1.5 mmol/kg)	100	92	92	92	92	92	92	92
N-Acetylcysteine (1.5 mmol/kg)	100	100	100	100	100	100	100	100
Paracetamol + *N*-acetylcysteine	100	100	100	100	100	92	92	92

Mice were dosed with benzo(*a*)pyrene (20 mg/kg, i.p.) for 3 consecutive days. Paracetamol (400 mg/kg, i.p.) was administered at the same time as CIPE (1.5 or 3.0 mmol/kg) or *N*-acetylcysteine (1.5 mmol/kg). Each treatment group consisted of at least six animals. Mortalities and body weight changes were monitored over 7 days as indicators of toxicity. The data represent the percentage animals surviving on each day.

contrast to our initial studies with uninduced mice when no toxicity was observed at this dose level. We therefore investigated the protective effects of a lower non-toxic dose of CIPE (1.5 mmol/kg). A marked protection against mortality associated with paracetamol intoxication was observed (Table 1). The protection afforded was comparable to that provided by *N*-acetylcysteine (1.5 mmol/kg) (Table 1). The surprising observation of an apparent increase in toxicity of CIPE in benzo(*a*)pyrene-induced mice appeared worthy of further investigation.

Effects of enzyme induction on the toxicity of CIPE

Pretreatment of mice with benzo(*a*)pyrene markedly increased the toxicity of CIPE. A dose-dependent mortality was observed; 1.5 mmol/kg caused no fatalities, while 3.0 and 6.0 mmol/kg caused 44 and 88% mortality, respectively, at the end of a 7-day observation period. There was also a dose-related loss and slowing in the body weight gain over 7 days compared to control animals (results not shown).

In marked constrast, neither control mice or mice induced with phenobarbitone suffered any mortalities after exposure to CIPE (1.5–6.0 mmol/kg). However, some effects were observed on body weight. At the highest dose (6.0 mmol/kg) of CIPE in control mice, a marked decrease in body weight was observed for several days. A similar but not as marked loss was noted in mice pretreated with phenobarbitone (results not shown).

DISCUSSION

Our studies demonstrate that CIPE caused a transient elevation in NPSH levels in the liver, lung, kidney and spleen (Fig. 1). The increase in hepatic NPSH, while short-lived, reduced the toxicity of paracetamol to mice induced with benzo(*a*)pyrene as measured by changes in NPSH, SGOT, SGPT and mortality (Figs 2 and 3 and Table 1). Preliminary data using HPLC suggest the elevation in mouse organ NPSH was due to an increase in intracellular cysteine, after hydrolysis of CIPE, in agreement with previous studies in the rat [25]. The very transient elevation in NPSH suggested that cysteine was rapidly metabolized. Cysteine may be utilized for GSH synthesis, oxidized to cystine or be metabolized by cysteine dioxygenase to cysteine sulphinate which may be further metabolized either by cysteine sulphinate decarboxylase, initiating a pathway leading to the formation of taurine or by glutamate-oxaloacetate transaminase, resulting in the formation of pyruvate and sulphite [32, 33]. Cysteine may also be metabolized by pathways independent of cysteine sulphinate including formation of 3-mercaptopyruvate with subsequent desulphuration [32, 33]. In our studies, no evidence was obtained for an increase in GSH up to 24 hr following CIPE administration (Fig. 2). Our results suggest that cysteine was rapidly metabolized, possibly by one of the above pathways, but under the conditions used in our studies was not utilized to any significant extent for GSH synthesis. This may have been due to the fact that as CIPE and paracetamol were administered together, GSH levels were sufficiently high to act as a feedback inhibitor of γ-glutamylcysteine synthetase, the rate-limiting step in the biosynthesis of GSH [34].

CIPE (1.5 mmol/kg) provided a marked protection against the toxicity of paracetamol (Table 1). CIPE (3.0 mmol/kg) also protected at early times but the results at later times were complicated by the toxicity of CIPE in mice induced with benzo(*a*)pyrene. The mechanism of this protection is not fully understood and there may be one or more possible explanations. The most likely is that CIPE or cysteine, derived

from hydrolysis, provide alternative nucleophilic centres to interact with reactive electrophiles so preventing the depletion of hepatic GSH below the critical threshold required for the induction of paracetamol hepatotoxicity (Fig. 2). Data from a variety of studies suggest that depletion of hepatic GSH below approximately 80% of control values is critical for the induction of the hepatotoxicity of a number of compounds including paracetamol [9] and bromobenzene [35]. When GSH is depleted below this critical level, the reactive electrophiles may then bind covalently to critical cellular macromolecules possibly leading to toxicity [36]. Alternatively, CIPE by increasing intracellular cysteine may alter paracetamol toxicity by increasing sulphate conjugation either by maintaining the supply of both the cofactor adenosine 3'-phosphate 5'-sulphatophosphate and also inorganic sulphate. Thiol compounds have been shown to interact directly with cytochrome P450, preventing the metabolic activation of paracetamol and inhibiting its covalent binding to hepatic mouse microsomes [37]. In these studies CIPE (1.5 mmol/kg) provided as good a protection against paracetamol-induced toxicity as *N*-acetylcysteine (Table 1), the compound most commonly used in the treatment of patients suffering from paracetamol overdose. Further studies are warranted on the chemoprotective potential of CIPE and a series of related cysteine esters [25] which are hydrolysed by esterases at different rates so potentially providing a more prolonged elevation in intracellular cysteine. These esters of cysteine may provide an alternative means of increasing intracellular cysteine compared to previous attempts using compounds such as OTZ [25].

An interesting complication of our study was the increased toxicity of CIPE in mice pretreated with benzo(*a*)pyrene compared either to controls or animals pretreated with phenobarbitone. The mechanism of this enhanced toxicity is not fully understood. CIPE alone following an initial elevation caused a prolonged decrease in hepatic NPSH (Fig. 2), possibly due in part to the formation of mixed disulphides. The enhanced toxicity following induction with benzo(*a*)pyrene is suggestive of an involvement of cytochrome P450 in the catabolism of CIPE/cysteine. This is somewhat surprising as cytochrome P450 is not generally considered to play a role in the metabolism of cysteine [32, 33]. However we cannot exclude the possibility that other enzymic activities such as esterase have been modified by the pretreatment with benzo(*a*)pyrene. The toxicity of cysteine is poorly understood, although it is known to damage the nervous system of new born rats [38].

In summary, CIPE causes transient elevation of NPSH in liver, lung, spleen and kidney and provides marked protection against paracetamol-induced mortality in mice.

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