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ELEVATION OF CYSTEINE AND REPLENISHMENT OF GLUTATHIONE IN RAT LUNG SLICES BY CYSTEINE ISOPROPYLESTER AND OTHER CYSTEINE PRECURSORS

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Abstract—In this study, we have used a rat lung slice model to compare the ability of several potential cysteine delivery systems (L-cysteine isopropylester, L-cysteine cyclohexylester, *N*-acetylcysteine, L,2-oxo-4-thiazolidine carboxylic acid and cysteine) to elevate cysteine and glutathione (GSH) levels in control lung slices and slices depleted of their GSH by diethyl maleate. The esters of cysteine produced the greatest rise in lung slice cysteine. All the cysteine delivery systems were capable of replenishing GSH in lung slices previously depleted of GSH by diethyl maleate.

Glutathione (GSH‡) is a tripeptide (γ-glutamylcysteinylglycine) with a wide biological distribution. GSH has several metabolic and protective functions central to cellular homeostatic control [1]. As a protectant, GSH can act as an antioxidant in conjunction with GSH peroxidase/reductase to protect against H_2O_2, organic hydroperoxide- or lipid hydroperoxide-mediated tissue damage [2], or alternatively it may conjugate with electrophilic molecules either spontaneously or in GSH *S*-transferase-mediated reactions [3].

In order to prevent oxidant- or electrophile-mediated injury, several methods have been proposed to raise or maintain cellular GSH levels during a toxicological insult. These include administration of GSH, cysteine, methionine, *N*-acetylcysteine, cysteamine, the cysteine precursor L,2-oxo-4-thiazolidine carboxylic acid (OTZ) and various esters of GSH [4–14].

A key factor determining the rate of GSH biosynthesis is the supply of L-cysteine [15], as cells are usually well supplied with glycine and glutamate, the other component amino acids of GSH. Intracellular cysteine levels can be influenced by uptake of γ-glutamylcysteine [16], or alternatively several cell types accumulate preformed cysteine and cystine using amino acid transport systems [17]. In order to overcome the problem of a limited supply of cysteine, a series of cysteine esters with increasing lipophilicity have been synthesized [18], including L-cysteine isopropylester (CIPE) and L-cysteine cyclohexylester (CCHE). These esters should lead to a rapid increase in intracellular cysteine following enzymic hydrolysis by esterases. This may be important in extrahepatic organs such as the lung which, unlike the liver [19], have little or no cystathionine pathway activity, so preventing their use of methionine as a cysteine precursor and making them largely dependent on preformed L-cysteine [20, 21].

CIPE and related esters have been shown to protect rats against the pulmonary oedema induced by perfluorisobutene, a pyrolysis product of Teflon [18], as well as against the hepatotoxicity of paracetamol [22]. It was demonstrated in both cases that following *in vivo* administration, CIPE produced a large but transient increase in pulmonary and hepatic non-protein sulphydryl (NPSH) which was due primarily to increases in cysteine [18, 22].

Depletion of pulmonary GSH by protein deficiency or diethyl maleate accentuated the injury to the lungs produced by oxidants [8], which may be alleviated by administration of thiols such as *N*-acetylcysteine and cysteamine [8, 23, 24]. Pulmonary GSH also plays a role in determining the outcome of intoxication with α-naphthylthiourea [25], naphthalene [26, 27] and 4-ipomeanol [28].

We wished to investigate the potential role of thiols, in particular cysteine and its derivatives, in protecting the lung against xenobiotic-induced damage. Cysteine or esters of cysteine may react directly with electrophiles or alternatively may replenish intracellular GSH so affording potential protection to the lung. We have concentrated on the lung because following CIPE administration, the elevation in tissue NPSH was most marked in this organ [22]. In this study, we have utilized rat lung slices in order to understand better the mechanisms of elevation of pulmonary NPSH. We have compared the abilities of CIPE and other cysteine delivery systems to elevate intracellular cysteine and GSH in both control and GSH-depleted lung slices.

We show that esters of cysteine produced the greatest sustained elevation in rat lung slice cysteine but did not improve the ability of the lung slice to replenish GSH after prior GSH depletion. The esters

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‡ Abbreviations: GSH, glutathione; CIPE, L-cysteine isopropylester; CCHE, L-cysteine cyclohexylester; OTZ, L,2-oxo-4-thiazolidine carboxylic acid; KRP, Krebs–Ringer phosphate buffer; NPSH, non-protein sulphydryl.

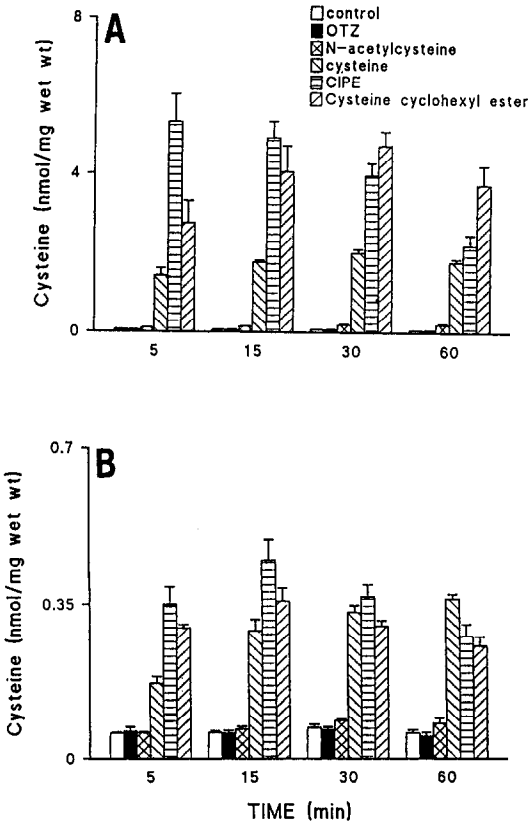

Fig. 1. The effect of several cysteine delivery systems on intracellular cysteine levels of rat lung slices. Lung slices were incubated at either (A) 500 or (B) 50 μM of the following compounds: control (□), OTZ (■), *N*-acetylcysteine (▨), cysteine (▤), CIPE (▥), CCHE (▧). Each bar represents the mean ± SEM (N = 3–8) analysed on separate occasions. The area under the curve for slices incubated with the cysteine esters (500 μM) is significantly greater than that for slices incubated with other delivery systems.

of cysteine probably protect against rapidly generated electrophiles by offering cysteine as an alternative nucleophilic centre to GSH, rather than supporting GSH biosynthesis.

MATERIALS AND METHODS

Compounds. CIPE and CCHE were synthesized at the Chemical and Biological Defence Establishment. L-Cysteine hydrochloride, *N*-acetylcysteine, OTZ and maleic acid diethyl ester (diethyl maleate) were obtained from the Sigma Chemical Co. (Poole, U.K.). *N*-Ethylmorpholine (>99% purity), methanesulphonic acid and bis(4-nitrophenyl)phosphate were obtained from the Aldrich Chemical Co. (Poole, U.K.). Monobromobimane (Thiolute®) was obtained from Calbiochem-Behring (San Diego, CA, U.S.A.).

Animals. Male Wistar rats (180–250 g) were supplied by Bantin and Kingman (Hull, U.K.). Animals were housed on a 12 hr light/dark cycle and allowed free access to food and water.

Preparation of rat lung slices. Rats were anaesthetized in a 3% halothane atmosphere and the lungs perfused with Krebs–Ringer phosphate buffer (KRP) (pH 7.4) supplemented with glucose until the lungs were clear of blood. Lung slices (0.5 mm thickness) were prepared using a McIlwain tissue chopper. To prepare lung slices with depleted GSH, slices (30–40 mg) were incubated with diethyl maleate (500 μM) (in dimethyl sulphoxide, 0.3%), for 30 min at 37° in 25 mL conical flasks shaken at 60 cycles/min. The slices were then washed in KRP, transferred to fresh KRP and incubated with various cysteine precursors. Control slices were treated in a similar manner but the addition of diethyl maleate was omittted. Diethyl maleate-pretreated slices were exposed to various cysteine delivery systems for up to 3 hr. Control slices were incubated with the delivery systems for 1 hr only. All incubation volumes were 3 mL.

Measurement of cysteine, GSH and esters of cysteine by HPLC. The measurement of lung sulphydryl content (cysteine, GSH, CIPE, CCHE and *N*-acetylcysteine) was made by HPLC analysis essentially as described [18]. Monobromobimane, which is membrane permeable, was used to derivatize pulmonary thiols and form fluorescent adducts.

After incubation with the various thiols, lung slices were briefly washed in KRP and immediately placed in 0.9 mL of monobromobimane (0.5 mg/mL, dissolved in 0.05% acetonitrile and 99.5% 0.05 M 4-ethylmorpholine, pH 8.0) and derivatized for 20 min. An aliquot of the incubating medium was also derivatized with monobromobimane. After 20 min, 0.1 mL of the protein precipitant methanesulphonic acid (10% v/v) was added to the derivatized lung slices and incubation medium. The lung slices were dispersed using a Polytron homogenizer. The majority of the excess, unconjugated monobromobimane was extracted from the homogenate by mixing with dichloromethane (0.5 mL) (Rathburn, U.K.) for 10 sec. After centrifugation, the aqueous layer was removed for HPLC analysis.

Samples were injected onto a Hypersil ODS 3 micron column (15 cm, 4.6 mm) (HPLC Technology, Macclesfield, U.K.) fitted with a C-18 guard column (μ-Bondapack C-18, Waters). The column was connected to a Waters 600E HPLC pump. Fluorescence was measured with a Waters 470 scanning fluorescence detector (excitation 390 nm, emission 477 nm). Derivatized samples were eluted at a flow rate of 1.3 mL/min with a discontinuous gradient, consisting of an initial step (12 min) with 8% (v/v) acetonitrile, 0.25% (v/v) acetic acid, pH 3.7, followed by step 2 (7 min) when the acetonitrile concentration was increased to 40% (v/v) and finally returning to the initial conditions for 6 min to requilibrate the column. For analysis of extracts obtained from CCHE-incubated rat lung slices, the gradient was stepped up to 70% acetonitrile, to reduce the retention time of CCHE on the column. Under these chromatographic conditions the following thiols were readily separated

with elution times: cysteine (4.7 min), GSH (14.7 min), CIPE (19.1 min) and CCHE (21.4 min).

Statistical analysis. Statistical significance was tested according to Student's *t*-test and the probability of a significant difference accepted when greater than 95% ($P < 0.05$).

RESULTS

Effect of CIPE and other cysteine precursors on the thiol levels of rat lung slices

When lung slices were incubated with CIPE or CCHE, little or no unmetabolized ester was detected. The intracellular ester levels achieved never exceeded 1% of the amount of cysteine ester added (data not shown).

The basal level of cysteine in the control lung slices was 0.059 ± 0.003 nmol/mg wet wt (59 μM), which was maintained during a 60 min incubation, in agreement with reported values for tissue concentration of cysteine (10–100 μM) [29]. CIPE or CCHE (500 μM) produced pronounced elevations of intracellular cysteine, in excess of the rise in pulmonary cysteine concentrations achieved with cysteine, *N*-acetylcysteine or OTZ (Fig. 1A). Elevation of cysteine by CIPE (500 μM) was rapid, reaching a maximum within 5 min. However, CCHE produced a maximal rise in pulmonary cysteine concentration at 30 min. The relative abilities of the different cysteine delivery systems to elevate intracellular cysteine were: CIPE = CCHE > cysteine > *N*-acetylcysteine > OTZ = control. When incubated at 50 μM, cysteine, CIPE and CCHE caused a similar elevation of intracellular cysteine. At 60 min, the cysteine concentration of slices incubated with cysteine (50 μM) alone was greater than the levels which resulted from incubation with CIPE or CCHE (Fig. 1B).

In contrast to the esters of cysteine, a pool of intracellular *N*-acetylcysteine was detected (0.034 ± 0.013 and 0.43 ± 0.09 nmol/mg wet weight for 50 and 500 μM, respectively, after 60 min incubation). *N*-Acetylcysteine produced only a small rise in intracellular cysteine compared to CIPE, CCHE or cysteine (Fig. 1A and B). OTZ could not be measured as it has no free- SH groups and consequently does not form a fluorescent conjugate with monobromobimane. Also, OTZ did not produce a detectable rise in intracellular cysteine in lung slices (Fig. 1). Slices incubated with cysteine (500 μM) reached their highest level of intracellular cysteine within 15 min which was maintained over the subsequent incubation period.

There was no difference in the profiles of intracellular cysteine between control lung slices and lung slices preincubated with diethyl maleate (data not shown).

Changes in extracellular cysteine, CIPE, CCHE and N-acetylcysteine mediated by rat lung slices

A time-dependent decrease in extracellular CIPE and CCHE (500 μM) was observed (Fig. 2), accompanied by a rise in extracellular cysteine (Fig. 3). Less than 10% of the unmetabolized esters remained after 60 min. The decrease in extracellular CCHE was slower than that in CIPE.

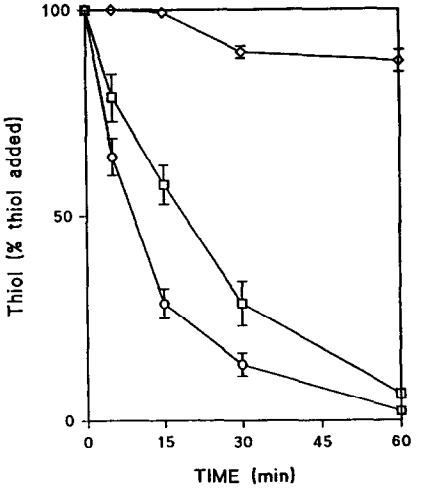

Fig. 2. Disappearance of CIPE (○) and CCHE (□) (500 μM) from the extracellular medium. *N*-Acetylcysteine (◇) (500 μM) is more resistant to hydrolysis by rat lung slices. The data are expressed as percentages of the amount of thiol added to the medium [initial sulphydryl levels (nmol/mL); CIPE, 521 ± 23.5; CCHE, 504 ± 35.4, *N*-acetylcysteine, 525 ± 29.3]. Each point represents the mean ± SEM of at least three separate experiments.

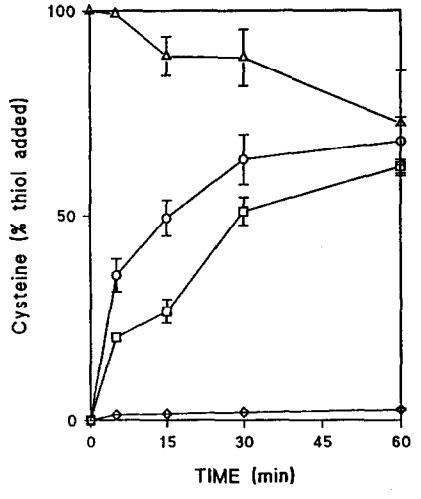

Fig. 3. CIPE (○) and CCHE (□) (500 μM) produce a steady rise in extracellular cysteine when incubated with lung slices. After 60 min, extracellular cysteine accounts for the majority of the R-SH initially added in the form of esters of cysteine. Cysteine (△) gradually declines over 60 min, while *N*-acetylcysteine (◇) leads to the generation of a small pool of cysteine. The data are expressed as percentages of the thiol initially added (initial levels of CIPE, CCHE and *N*-acetylcysteine are the same as in Fig. 2; initial level of cysteine, 582 ± 43.3 nmol/mL). Each point represents the mean ± SEM of at least three separate experiments.

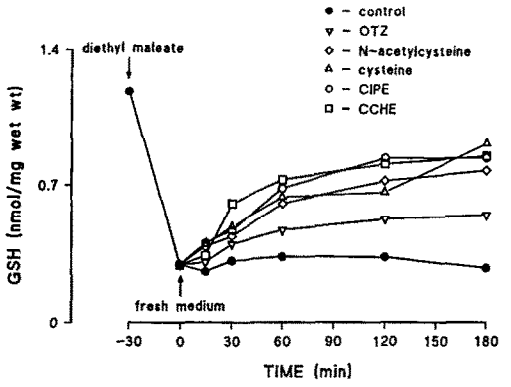

Fig. 4. Ability of various cysteine delivery systems to replenish GSH in GSH-depleted rat lung slices. Incubation of rat lung slices with diethyl maleate (500 μM) for 30 min resulted in a decrease of 75% in GSH levels. These slices were then transferred to fresh KRP either alone (●) or in the presence of various cysteine delivery systems (all at 500 μM) as indicated in the Figure. Each point is the mean ± SEM of at least four separate experiments. All the cysteine delivery systems resulted in a partial replenishment in pulmonary GSH, the area under the curve being significantly different from controls. No difference in intracellular GSH existed between slices incubated with cysteine, *N*-acetylcysteine, CIPE or CCHE. The error bars have been omitted from the Figure for the sake of clarity, but in all cases they were ≤10% of the mean.

The extracellular pool of *N*-acetylcysteine (500 μM) remained relatively constant over 60 min (Fig. 2). A limited rise in extracellular cysteine was noted (Fig. 3).

After 60 min incubation of rat lung slices with cysteine (500 μM), extracellular cysteine declined by 21% (Fig. 3). When cysteine was incubated in the absence of lung slices there was no appreciable decline in cysteine over 60 min (data not shown). OTZ had no effect on extracellular thiol levels.

A similar pattern of extracellular thiols was observed with lower concentrations of the cysteine prodrugs (50 μM). A comparison of extracellular thiols from slices preincubated with diethyl maleate showed a similar trend to control medium.

Effect of CIPE and other cysteine precursors on the GSH levels of rat lung slices

Control lung slices had initial GSH levels of 1.23 ± 0.12 nmol/mg wet weight which were maintained over a 3 hr incubation period. These values are in good agreement with published data [25, 26, 30]. No elevation of pulmonary GSH was observed in control lung slices incubated with CIPE, CCHE, *N*-acetylcysteine, OTZ or cysteine (all 500 μM) for up to 2 hr (data not shown).

Incubation of rat lung slices with diethyl maleate (500 μM) for 30 min caused a 75% fall in pulmonary GSH. GSH levels did not recover when slices were incubated in fresh KRP for a further 3 hr (Fig. 4).

Interestingly, in GSH-depleted lung slices, all five cysteine delivery systems supported resynthesis of GSH to varying extents (Fig. 4). The greatest increases were observed with CIPE, CCHE, *N*-acetylcysteine and cysteine, whilst OTZ was apparently less effective (Fig. 4).

DISCUSSION

The two esters of cysteine were particularly efficient cysteine delivery systems, producing a rapid burst in intracellular cysteine. The elevated levels of cysteine were fairly short lived (Fig. 1), as the esters were metabolized and removed from the extracellular pool. Cysteine added either exogenously or derived from hydrolysis of the esters is probably accumulated into lung cells by neutral amino acid uptake systems [17]. The esters of cysteine probably act independently of these systems, as the initial increases in intracellular cysteine were greater than those observed when lung slices were incubated solely with cysteine (Fig. 1). The loss of intracellular cysteine following its generation from the cysteine delivery systems may be due to one or more possibilities. The data presented suggested that much of the cysteine was exported from lung slices. Once free from the reducing environment of the slice, oxidation of cysteine to cystine would account for the gradual disappearance of cysteine from the extracellular pool. Alternatively, the intracellular cysteine may contribute to the turnover of pulmonary GSH [21], or may possibly undergo catabolic metabolism, ultimately generating taurine or pyruvate and sulphite [31]. However, the majority of studies on the metabolic fate of cysteine have been carried out with mammalian liver and may not be applicable to its fate in the lung.

Differences were observed in intracellular cysteine profiles following exposure to the esters of cysteine. CIPE produced a rapid increase in cellular cysteine, with maximum levels being observed within 5 min, whilst CCHE produced a slower but more sustained elevation in intracellular cysteine (Fig. 1). This corresponded to the longer half-life of CCHE in the extracellular medium and may have been due to a slower hydrolysis by pulmonary esterases caused by the bulkier cyclohexyl group.

In control lung slices, none of the cysteine precursors produced an increase in GSH, most probably because of a negative feedback on γ-glutamylcysteine synthetase by normal levels of GSH [32]. All the cysteine delivery systems were capable of replenishing lung slice GSH after exposure to diethyl maleate (Fig. 4). The largest increases in GSH occurred after 1 hr incubation, when the majority of the esters had already disappeared (Fig. 2) and intracellular cysteine levels for ester-incubated slices approached levels experienced by slices incubated with cysteine alone. This may account for the failure of the cysteine esters to offer any advantage over cysteine in the replenishment of GSH.

N-Acetylcysteine and OTZ were relatively ineffective in elevating intracellular cysteine in control lung slices (Fig. 1), but both promoted GSH generation in diethyl maleate-treated slices (Fig. 4). In *N*-acetylcysteine-incubated slices, cysteine levels were consistently below 0.3 mM, the reported K_m value of cysteine for renal and erythrocyte γ-

glutamylcysteine synthetase [33]. As the lung is a heterogeneous organ, the location of pulmonary GSH synthesis supported by *N*-acetylcysteine or OTZ may be limited to cells possessing deacetylase or prolinase activity. Whilst the apparent intracellular cysteine concentration for lung slices is below the K_m value for γ-glutamylcysteine synthetase, cellular compartmentation could account for this anomaly. Lung homogenates deacetylate *N*-acetylcysteine [34] but the ability of the lung to use OTZ for the generation of cysteine is variable [29]. Several of the cysteine precursors studied have been shown previously to slow the depletion of GSH after a toxic insult and support GSH resynthesis. CIPE, OTZ and *N*-acetylcysteine protect against the hepatotoxicity of paracetamol [22, 29, 35]. *N*-Acetylcysteine increases the biosynthesis of GSH in isolated perfused lungs [20] and when administered in the drinking water increases pulmonary GSH 2-fold [36]. *N*-Acetylcysteine has also been proposed to have a role in treating human pulmonary disorders, being a muceolytic agent [37]. Preliminary results show *N*-acetylcysteine may be beneficial in adult respiratory distress syndrome [38].

In summary, the esters of cysteine produced a marked elevation of cysteine in rat lung slices compared to cysteine or *N*-acetylcysteine, but without offering any advantage in their ability to replenish pulmonary GSH. The esters of cysteine have been observed to protect against rapidly generated electrophiles, such as inhaled perfluoroisobutene in rats pretreated with L-buthionine sulphoximine [18] and paracetamol in mice with induced cytochrome P450 activity [22]. The protection observed is probably associated with the rapid burst in intracellular cysteine [18, 22]. The cysteine may act as an alternative focus for electrophilic attack, sparing GSH and preventing interaction with other critical nucleophilic sites, rather than replenishing GSH, the proposed mechanisms by which *N*-acetylcysteine protects against paracetamol intoxication [39]. The esters of cysteine may prove to be less effective against xenobiotic compounds, which induce a prolonged release of electrophiles.

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