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COMPARISON OF THE DELIVERY OF REDUCED GLUTATHIONE INTO P388D$_1$ CELLS BY REDUCED GLUTATHIONE AND ITS MONO- AND DIETHYL ESTER DERIVATIVES

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Abstract—The effect of reduced glutathione, reduced glutathione monoethyl ester and reduced glutathione diethyl ester on the cellular concentration of reduced glutathione and cysteine in P388D$_1$ macrophages *in vitro*, and the cellular and extracellular de-esterification of reduced glutathione esters, was investigated. At 1 mM reduced glutathione derivative, only reduced glutathione diester markedly increased the cellular concentration of reduced glutathione. There was little delivery of reduced glutathione monoethyl ester into the cells. Reduced glutathione, and monoethyl and diethyl ester derivatives all increased the cellular concentration of cysteine; reduced glutathione diethyl ester also increased the cellular concentration of γ-glutamylcysteine. Reduced glutathione esters were de-esterified intracellularly where the diester was rapidly converted to the monoester. The diester was also converted to the monoester extracellularly by interaction with cell surface esterases and by a much slower spontaneous hydrolysis. This indicates that the diester of reduced glutathione was a much more effective vehicle for delivery of reduced glutathione into cells than the monoester. Reduced glutathione diester also increased the cellular concentrations of cysteine and γ-glutamylcysteine, suggesting that *de novo* synthesis of reduced glutathione was also stimulated.

Key words: reduced glutathione; glutathione monoester; glutathione diester; P388D$_1$ cells; macrophage; oxidative stress

The decrease of the cellular concentration of reduced glutathione (GSH†) on exposure to toxicants and ionizing radiation is implicated in the development of cytotoxicity [1–3]. The cellular concentration of GSH is decreased in oxidative stress induced by peroxides [4], redox-cycling quinones [5] and ionizing radiation [6], by alkylating agents, other electrophilic compounds and reactive metabolites [7], and by inhibition of γ-glutamylcysteine synthetase [8] and glutathione reductase [9]. Severe depletion of the cellular concentration of GSH leads to enzyme inactivation and protein denaturation [10, 11], lipid peroxidation [12] and oxidative degradation of nucleic acids [13], including, in some cases, induction of apoptosis [14]. Pharmacologic repletion of cellular GSH may prevent this toxicity.

Repletion of the cellular levels of GSH may be achieved by addition of *N*-acetyl-L-cysteine [15], cysteine esters [16], L-2-oxothiazolidine-4-carboxylic acid [17] and methionine [18] which supplement the supply of cysteine to cells required for *de novo* synthesis of GSH. The direct supplementation of the cellular pool of GSH by esters of GSH [19–21] has also proven effective and is preferable because: (i) it circumvents the requirement for ATP in the *de novo* synthesis of GSH—ATP is also depleted in cells suffering toxic insult [22], and (ii) it by-passes the γ-glutamylcysteine synthetase step in the *de novo* synthesis of GSH which is feedback inhibited by GSH [23]. The circumvention of the feedback inhibition of γ-glutamylcysteine synthetase is expected to be of particular importance for prophylactic administration of GSH ester to produce GSH loading of cells but may not be as important in toxicant stress *in vivo* where, by definition, the concentration of GSH is decreased below normal values before antidote therapy is initiated. Moreover, in mild toxicant stress, GSH alone can circumvent γ-glutamylcysteine synthetase by extracellular formation of γ-glutamylcystine, delivery of this into cells and thiol-disulphide exchange to form γ-glutamylcysteine [24].

The monoethyl ester derivative of GSH, γ-glutamylcysteinyl-ethylglycinate (GSHEt), was initially proposed as a vehicle for delivery of GSH into cells [19]. Acid-catalysed esterification of GSH leads to the formation of two forms of monoester, the γ-glutamyl and glycyl monoesters, and a diester derivative [20]. The γ-glutamyl monoester derivative, γ-(α-ethyl)glutamyl-cysteinylglycine, hydrolysed spontaneously during purification and is probably related to the neighbouring amino group of the γ-glutamyl residue [20]. Following the discovery that only the diester derivatives and not monoester derivatives of glutathione-*S*-conjugates were effec-

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† Abbreviations: GSH, reduced glutathione; GSHEt, reduced glutathione monoethyl ester; GSHEt$_2$, reduced glutathione ethyl diester; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(1-ethane-sulphonic acid); EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propane-sulphonic acid); MGB, modified Gey's buffer.

tively delivered into cells [25], the diester derivative of GSH, γ-(α-ethyl)glutamylcysteinyl-ethylglycinate ($GSHEt_2$), was also found to deliver GSH into cells more effectively than the monoester [21].

A further factor in the efficacy of delivery of GSH into cells as ester derivatives is the hydrolysis of the ester groups in the plasma and extracellular medium. It has been reported that GSH diester was unreactive to hydrolysis by human and guinea pig plasma esterases but reactive towards rat and murine plasma esterases [21]. A further esterase activity which was not considered is that located on the extracellular surface of the plasma membrane.

In this report, we describe the delivery of GSH into $P388D_1$ macrophages by GSH and its mono- and diethyl ester derivatives. We conclude that GSH diester was a markedly better vehicle for delivery of GSH into cells than the monoester, and there was significant extracellular de-esterification of the diester. Unexpectedly, GSH diester also increased the cellular concentration of both cysteine and γ-glutamylcysteine, suggesting the *de novo* synthesis of GSH was stimulated.

MATERIALS AND METHODS

Materials. GSH, Chelex resin (sodium form), monobromobimane, D-penicillamine, L-cysteine, γ-L-glutamyl-L-cysteine (trifluoroacetate salt), EPPS and sulphosalicylic acid were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, U.K.). GSHEt and $GSHEt_2$ were prepared and purified as described [20, 21] and characterized by $[^1H]$ and $[^{13}C]$NMR spectroscopy, FAB mass spectrometry, thin layer chromatography and analytical reverse phase HPLC. Nova-Pak octadecylsilica (ODS) 4 μM (0.8 cm × 10 cm) cartridges and pre-columns (8 cm × 10 cm) for HPLC were purchased from Waters–Millipore (Watford, U.K.). RPMI 1640 cell culture medium and foetal calf serum were purchased from Gibco Europe Ltd (Paisley, Scotland).

The murine macrophage $P388D_1$ cell line [26] was purchased from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, U.K.). A colony of $P388D_1$ cells was maintained by seeding at a density of 80,000 cells/mL in RPMI 1640 with 10% foetal calf serum and 2 mM glutamine in a 75 cm^2 T-flask under an atmosphere of air with 5% carbon dioxide, 100% humidity and 37° under aseptic conditions. The cells were cultured for 4 days to confluence. They were harvested by scraping adherent cells free and collected by centrifugation (400 *g*, 5 min). The cells were resuspended in fresh culture medium (1 mL), counted and cell viability assessed by the Trypan blue exclusion technique [27]. A portion of the cells was used to maintain the colony; the remainder were sedimented by centrifugation (400 *g*, 5 min), resuspended in modified Gey's buffer (MGB) containing 147 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 1.5 mM $CaCl_2$, 0.3 mM $MgSO_4$ and 1 mM $MgCl_2$, pH 7.4 and 37° and used in incubations with the GSH, GSHEt and $GSHEt_2$ [4].

Delivery of reduced glutathione into $P388D_1$ macrophages by glutathione esters. $P388D_1$ cells (2×10^6) were incubated in MGB (1 mL) with and without 1 mM or 10 mM GSH, GSHEt and $GSHEt_2$ for 3 hr at pH 7.4 and 37°. At the times indicated, the cells were collected by centrifugation (2300 *g*, 2 min) and an aliquot (100 μL) of the supernatant removed and retained. The cell pellet was washed once with 1 mL of ice-cold MGB. Both the supernatant and cell pellet were analysed for non-protein thiols (see below).

The possible activation of the respiratory burst in $P388D_1$ cells by exposure to GSH, GSHEt and $GSHEt_2$ was investigated by measuring the activity of the NADPH oxidase in $P388D_1$ cells (2×10^6/mL) incubated with and without 1 mM GSH, GSHEt and $GSHEt_2$ for 1.5 and 3.0 hr in MGB at 37° [28]. No activation of NADPH oxidase by the GSH derivatives was found, suggesting that $P388D_1$ cells were not activated by GSH or GSH esters.

The stability of the GSH derivatives in MGB was studied by incubation of GSH, GSHEt and $GSHEt_2$ (1 mM) in MGB at 37° for 3 hr and analysis of the remaining thiol-containing compounds.

The volume of a $P388D_1$ cell was determined by preparing a cell pellet of *ca.* 20×10^6 cells, removing the supernatant, and sampling 10 μL of packed cells from within the cell pellet with a positive displacement pipette. The cells were then resuspended in 10 mL of MGB and counted on a haemocytometer. The $P388D_1$ cell volume given is the mean ± standard deviation of three independent determinations.

Assay of non-protein thiol compounds. GSH, GSHEt, $GSHEt_2$, cysteine and γ-glutamylcysteine were assayed in $P388D_1$ cells and extracellular medium by derivatization with monobromobimane and separation of the adducts by reversed phase HPLC [21, 29]. The internal standard was D-penicillamine. The concentrations of the thiol-containing analytes were determined by reference of analyte/internal standard integral ratios to a standard curve produced with authentic compounds.

$P388D_1$ cells (2×10^6) in MGB (100 μL) or supernatant (100 μL) from the incubations with GSH derivatives were treated with 5% (w/v) sulphosalicylic acid (40 μL) and vortex mixed. D-Penicillamine (1 mM, 10 μL) and monobromobimane (100 mM, 10 μL) were added. The derivatization reaction was initiated by addition of EPPS buffer, pH 8 (1 M, 160 μL). The final concentration of monobromobimane in the incubation was 3.13 mM which was a greater than 9-fold excess over the non-protein thiol concentration. The reaction mixture was left at room temperature in the dark for 10 min and then further reaction was quenched by addition of glacial acetic acid (20 μL). The derivatization mixture was filtered (0.2 μM) and an aliquot (50 μL) analysed by reversed phase HPLC. HPLC was performed with a Waters HPLC system (2 × 510 pumps, Lambda Max 481 LC spectrophotometer with a 680 automated gradient controller). The column was Nova-Pak octadecylsilica 4 μM (0.8 × 10 cm) cartridge fitted with a pre-column in an 8 × 10 radial compression unit. The mobile phase was: solvent A (850 mL water, 150 mL methanol, 2.5 mL glacial acetic acid, pH 3.9) and solvent B (100 mL water, 900 mL of methanol, 2.5 mL acetic acid, pH 3.9). The solvent programme was 0–5 min 100% solvent A, and 5–45 min a linear gradient of 0–50% solvent B, with a

Table 1. [^{13}C]NMR spectroscopy of glutathione monoethyl and diethyl esters in 100 mM NaD_2PO_4/Na_2DPO_4 in D_2O, pH 7.4

Compound	Monoester	Diester
Assignment		
Glycyl		
C-1	174.3	174.6
C-2	40.8	41.4
Cysteinyl		
C-1	170.0	171.0
C-2	55.0	55.6
C-3	24.7	25.3
γ-Glutamyl		
C-1	172.1	172.0
C-2	53.4	52.4
C-3	25.4	26.5
C-4	30.5	30.8
C-5	173.3	172.6
Other		
	Ethyl (Gly)	Ethyl (Gly)
	C-1 62.0	C-1 63.1
	C-2 12.7	C-2 13.2
		Ethyl (Glu)
		C-1 62.5
		C-2 13.2

Chemical shift δ_c values (ppm) for spectra recorded at 68 MHz of reduced glutathione derivatives (10 mg/mL).

flow rate of 2 mL/min. The eluate was monitored by flow spectrophotometry at 390 nm (a fluorescence detector was not available to us); all analyte peaks were resolved from monobromobimane degradation peaks and other products. The retention times of the derivatized thiol compounds and metabolites were: cysteine 4.0 min, γ-glutamylcysteine 5.5 min, GSH 7.5 min, D-penicillamine 15.0 min, GSHEt 23.0 min, and $GSHEt_2$ 35.0 min. The limits of detection of the thiol compounds were defined as the concentration of analyte equal to integral ratio of the zero control plus two standard deviations, as determined from the calibration curve. The detection limit values for the thiol analytes in the $P388D_1$ cell extracts (nmol/10^6 cells) were: cysteine 0.23, γ-glutamylcysteine 0.27, GSH 0.15, GSHEt 0.17 and $GSHEt_2$ 0.23. There were also peaks in the chromatogram due to monobromobimane degradation products with retention times of 11.5, 24.9, 31.5 and 33.5 min.

RESULTS

Characteristics of reduced glutathione monoethyl ester and reduced glutathione diethyl ester

GSHEt and $GSHEt_2$ were prepared and purified as previously described [20, 21]. They were characterized by [^{1}H] and [^{13}C]NMR spectroscopy—the characteristics of the [^{1}H]NMR spectra were as given previously [20]; the characteristics of the [^{13}C]NMR spectra have not been reported and are given in Table 1. FAB mass spectrometric analysis gave the expected molecular ion M + 1 peaks of *m/z* values 336 and 364 for GSHEt and $GSHEt_2$, respectively. Since the characteristics of GSH delivery into cells by the two compounds were to be distinguished, assessment of the purity of the preparations was important. Purity was assessed by analytical reversed phase HPLC of the underivatized peptides with absorbance detection of eluate at 220 nm, with a mobile phase of 10 mM sodium phosphate buffer, pH 2.83, and a linear methanol gradient of 0–50% methanol over 20 min, as previously described [20]. GSHEt gave a single peak in the chromatogram of retention time 9.8 min, and $GSHEt_2$ gave a single peak of retention time 15.0 min.

Delivery of reduced glutathione into $P388D_1$ cells by esters of reduced glutathione

The concentration of GSH and cysteine in $P388D_1$ cells was 4.08 ± 0.20 nmol/10^6 cells and 0.80 ± 0.06 nmol/10^6 cells, respectively, and did not change significantly during incubation for 3 hr in MGB at 37°. There was the appearance, however, of a low concentration of GSH in the culture medium after the 3 hr incubation (Table 2).

When $P388D_1$ cells (2×10^6/mL) were incubated in MGB, pH 7.4 and 37°, for 3 hr in the presence of 1 mM GSH, the cellular concentration of GSH was increased by 11% of the control value ($P < 0.02$). There was also a 29% increase in the cellular concentration of cysteine, relative to the control value ($P < 0.001$) (Fig. 1). The concentration of GSH in the incubation medium decreased by *ca.* 29% during the 3 hr incubation and cysteine was now detected in the extracellular medium; the cysteine concentration in the extracellular medium of control incubations was below the limit of detection, <0.45 μM (Table 2). The decrease in the concentration of GSH in the extracellular medium was greater than the decrease observed when GSH was incubated in MGB at 37° in the absence of cells but the difference was not significant—294 ± 53 μM versus 283 ± 96 μM ($P > 0.05$; $N = 3$). In the absence of cells, GSH degraded by autoxidation to non-monobromobimane-reactive (non-thiol) compounds.

When $P388D_1$ cells (2×10^6/mL) were incubated for 3 hr in the presence of 1 mM GSHEt, the cellular concentration of GSH was increased by 10% of the control value but this increase was not significant ($P > 0.05$). There was a low concentration of GSHEt in the cells, 0.28 ± 0.05 nmol/10^6 cells ($N = 3$). There was, however, a marked rise in the cellular concentration of cysteine which was increased by 139% of control values to 1.91 ± 0.05 nmol/10^6 cells ($P < 0.001$, $N = 3$) (Fig. 1). During the 3 hr incubation, the concentration of GSHEt in the cell supernatant decreased by 20% of its initial value. There were also increased concentrations of cysteine and GSH in the supernatant (Table 2). The decrease in the concentration of GSHEt in the cell supernatant was markedly lower than the decrease observed for the incubation of GSHEt in MGB at 37° in the absence of $P388D_1$ cells—196 ± 62 μM versus 643 μM ± 25 ($P < 0.001$; $N = 3$). In the absence of cells, GSHEt degraded by autoxidation to non-monobromobimane-reactive (non-thiol) compounds.

Table 2. Reduced glutathione derivatives and cysteine in the extracellular medium of P388D$_1$ macrophages incubated with reduced glutathione, reduced glutathione monoester and reduced glutathione diester

Addition	Concentration of thiol compound (μM) mean ± SD (N = 3)			
	Cysteine	GSH	GSHEt	$GSHEt_2$
Control	<0.45	3.60 ± 1.47	—	—
+ GSH (1 mM)	3.32 ± 0.71	705.96 ± 52.79	—	—
+ GSHEt (1 mM)	20.37 ± 15.02	10.28 ± 0.66	804.06 ± 62.37	—
+ $GSHEt_2$ (1 mM)	5.55 ± 3.13	7.20 ± 3.01	136.54 ± 10.17	570.00 ± 53.95

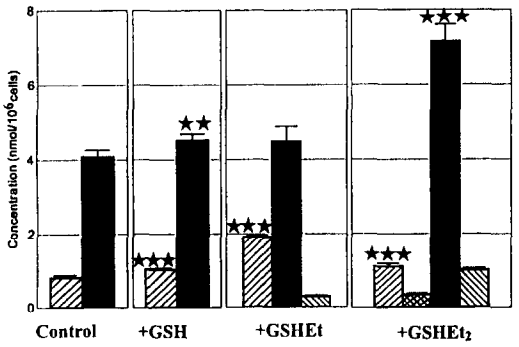

Fig. 1. The concentration of non-protein thiol metabolites in P388D$_1$ macrophages incubated with 1 mM GSH, GSHEt and $GSHEt_2$. P388D$_1$ macrophages (2×10^6/mL) were incubated in MGB, pH 7.4 and 37°, for 3 hr in the presence of the glutathione derivative indicated. The concentration of non-protein thiol was determined as described in Materials and Methods. Data are mean ± SD of three determinations. Key: (▨) cysteine, (▩) γ-glutamylcysteine, (■) GSH, (▧) GSHEt. Significance tests: GSH and cysteine concentrations with respect to control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

When P388D$_1$ cells (2×10^6/mL) were incubated for 3 hr with 1 mM $GSHEt_2$, the cellular concentration of GSH increased by 75% of control values to 7.13 ± 0.45 nmol/10^6 cells (N = 3). There was also a relatively high concentration of GSHEt in the cells: 1.00 ± 0.05 nmol/10^6 cells (N = 3). $GSHEt_2$ was however, undetectable in the P388D$_1$ cells (<0.23 nmol/10^6 cells) under these conditions. This unexpected finding is attributed to rapid cytosolic hydrolysis of reduced glutathione diester to the monoester. The cellular concentration of cysteine was increased markedly, by 165% of control values, and γ-glutamylcysteine was also detected (Fig. 1). In the extracellular medium, the concentration of $GSHEt_2$ decreased by 43% with the concomitant appearance of GSHEt, GSH and cysteine (Table 2). The decrease in the concentration of $GSHEt_2$ in the extracellular medium was lower than the decrease in $GSHEt_2$ incubated in MGB in the absence of P388D$_1$ cells—430 ± 54 μM versus 510 ± 48 μM (N = 3) but the difference was not significant ($P > 0.05$). In MGB in the absence of cells, $GSHEt_2$ degraded by spontaneous hydrolysis to the GSHEt and autoxidation to non-thiol-containing compounds; for an initial concentration of $GSHEt_2$ of 1 mM, after incubation for 3 hr in MGB at 37° the concentration of GSHEt was 103.0 ± 4.0 μM, equivalent to *ca.* 20% of the $GSHEt_2$ degradation.

The delivery of $GSHEt_2$ into P388D$_1$ cells was observed when cells were incubated with 10 mM $GSHEt_2$ in MGB at 37° for 1 hr. Under these conditions, the concentrations of metabolites (nmol/10^6 cells) were: cysteine 6.76 ± 0.37, γ-glutamylcysteine 6.58 ± 0.58, GSH 9.81 ± 0.79, GSHEt 17.84 ± 0.18 and $GSHEt_2$ 1.04 ± 0.71 (N = 3). The concentration of GSH was increased by 141% above control values, and there was a net increase of cellular reduced glutathione derivatives (GSH, GSHEt, $GSHEt_2$) of 604%. There were also marked increases in the cellular concentration of cysteine and γ-glutamylcysteine. Addition of GSH derivatives at 10 mM was not used for the comparison of the efficacy of GSHEt and $GSHEt_2$ since 10 mM GSHEt gave a 19% decrease in viability of P388D$_1$ cells ($P < 0.001$) under these conditions.

A study of the metabolites of $GSHEt_2$ in P388D$_1$ cell cultures over a 3 hr incubation period showed the appearance of GSHEt in the P388D$_1$ cells and increase in the concentration of GSH and cysteine after only 5 min of incubation (Fig. 2). These increases continued until 30–60 min. Thereafter, the concentrations of GSH and cysteine continued to increase slowly but the concentration of reduced glutathione monoester did not change significantly. The concentration of γ-glutamylcysteine increased during the incubation but was only above the limit of detection after 3 hr when the concentration was 0.34 ± 0.03 nmol/10^6 cells.

DISCUSSION

GSH does not readily cross cell membranes, although intestinal epithelial cells [30], kidney epithelial cells of the basolateral membrane [31] and alveolar type II epithelial cells [32] have a sodium-dependent GSH uptake system. There is, however, slow leakage of GSH out of cells from the cytosol which may occur by the recently characterized bidirectional GSH transporter [33]. Under physiological conditions, there is little entry of GSH into

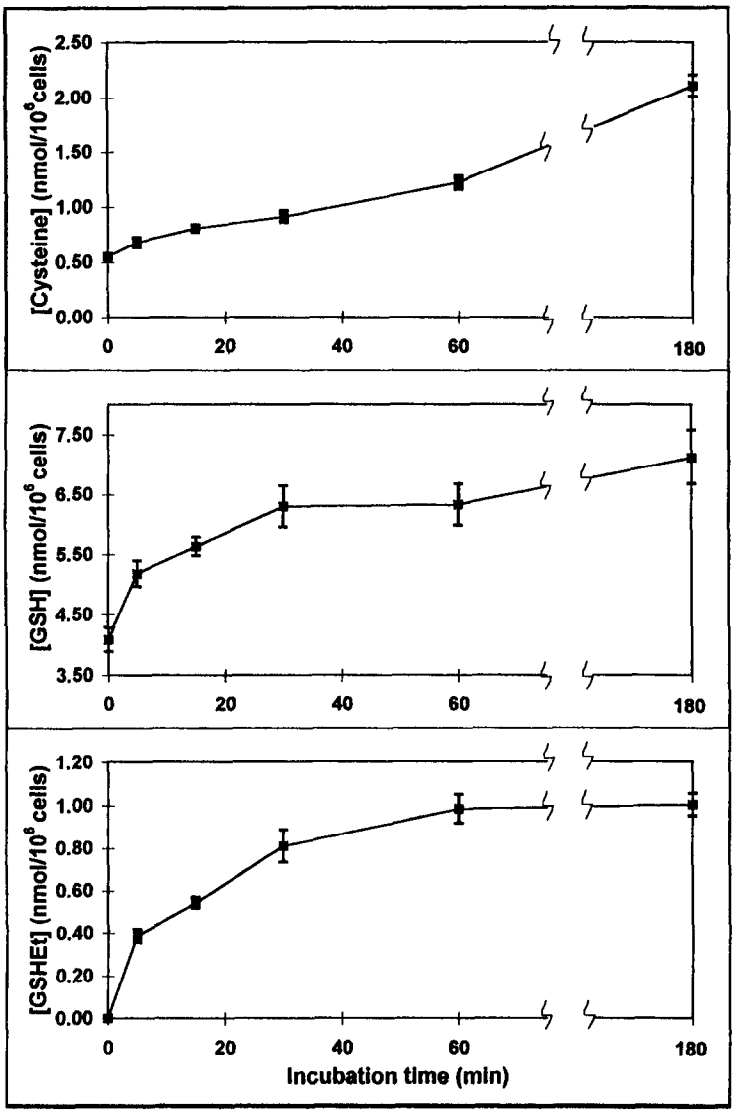

Fig. 2. Time course of the concentration of non-protein thiol metabolites in P388D$_1$ macrophages incubated with 1 mM $GSHEt_2$ in MGB, pH 7.4 and 37°. The concentration of non-protein thiol was determined as described in Materials and Methods. Data are mean ± SD of three determinations.

cells, attributed to the low extracellular concentration of GSH (due to degradation by cell surface-localized γ-glutamyl transferase and dipeptidase), and the high K_m value (2.4 mM) of GSH for the transporter [33]. GSH does not normally cross cell membranes by passive diffusion because all the major solution forms of GSH have ionized groups. This also applies to GSHEt. Reduced glutathione diester, however, has a major solution species which is uncharged (the form where the γ-glutamyl α-amino group is in the amino $-NH_2$ form and the cysteinyl sulphydryl group is in the thiol –SH form) which may give rise to efficient and effective membrane transport. This was initially recognized and observed for the delivery of glutathione-*S*-conjugates into cells by diesterification [25] and subsequently observed for $GSHEt_2$ [21]. Although the esters of GSH are unreactive to removal of the γ-glutamyl group by γ-glutamyl transferase [19, 21], the ester group may be hydrolysed by plasma and/or cell surface-localized esterases.

Extracellular GSH is degraded by γ-glutamyl transferase bound on the external surface of cell membranes; *in vivo*, the enzymatically active subunit may also be present in blood plasma [34]. The reaction of GSH with γ-glutamyl transferase *in vivo* was thought, until recently, mainly to involve hydrolysis to form glutamate and cysteinylglycine [35]; cysteinylglycine is then hydrolysed to cysteine and glycine by dipeptidase on the external surface of the plasma membrane. Cysteine may enter cells by the sodium-dependent ASC transporter, which also transports cysteine for export from the cell cytosol [36]. In the extracellular medium of cells,

however, cysteine may rapidly oxidize to cystine [37]. This may enter cells by the X_c^- transporter in exchange for glutamate [36]. Recent studies, however, have implicated γ-glutamyl transferase activity in the uptake of cystine by cells where γ-glutamylcystine is formed extracellularly from cystine and GSH: γ-glutamylcystine is then taken into cells by the γ-glutamyl amino acid transporter, and in the cell cytosol is reduced to γ-glutamylcysteine and cysteine by GSH [24, 38]. Thereby, γ-glutamylcysteine may be formed in cells by a γ-glutamylcysteine synthetase-independent mechanism.

Incubation of $P388D_1$ cells with GSH (1 mM) gave a small significant increase in the cellular concentration of GSH and an increase in the concentration of cysteine. The small increase in GSH concentration may have arisen from an increase in *de novo* synthesis of GSH stimulated by the supply of component amino acids from the degradation of GSH in the extracellular medium by membrane-bound γ-glutamyl transferase and dipeptidase; feedback inhibition of γ-glutamylcysteine synthetase by GSH may prevent further increase. There was a concomitant increase in the extracellular concentration of cysteine consistent with this. There may also be a contribution from the bidirectional GSH transporter which, with 1 mM GSH in the extracellular medium, is expected to be functional for GSH entry into cells [33].

Incubation of $P388D_1$ cells with GSHEt (1 mM) gave a small increase in the cellular concentration of GSH but the increase was not significant. There was, however, the appearance of a low concentration of GSHEt in $P388D_1$ cells at an estimated cellular concentration of 0.28 nmol/10^6 cells or 7% of the control concentration of GSH. There was a more marked increase in the cellular concentration of cysteine in GSHEt-treated cells. This may be due to both uptake of cysteine and cystine (formed by spontaneous oxidation of cysteine). The initial step of the extracellular degradation of GSHEt is expected to be de-esterification by membrane-bound esterase since the monoester is unreactive to cleavage by γ-glutamyl transferase [19]. Consistent with this was the presence of GSH in the extracellular medium. $P388D_1$ cells had a stabilizing effect on GSH monoester. This may be due to the chelation of trace redox-active metal ions, catalysts of the autoxidation of GSHEt, by cell membrane proteins and secreted proteins.

The prevention of toxicity by GSHEt has been demonstrated in drug-induced [39] and radiation-induced toxicity [40]. The expression of human immunodeficiency virus was also suppressed by GSH monoester [41]. In these studies, the effects may have been mediated by the increase in the cellular concentration of cysteine. The method used to prepare reduced GSH monoester in these and other studies [42] gave GSH monoester preparations of questionable purity where an expected contaminant was GSH diester [20]. Contamination with GSH diester can now be seen to compromise the validity of the antidote effects of some GSH monoester preparations.

Incubation of $P388D_1$ cells with $GSHEt_2$ markedly increased the cellular concentration of GSH: with 10 mM $GSHEt_2$, after 1 hr the cellular concentration of GSH was 9.82 nmol/10^6 cells or *ca.* 5.66 mM (*cf.* control values of 4.08 nmol/10^6 cells or 2.35 mM). The volume of a $P388D_1$ cell was determined and found to be $2.5 \pm 0.2 \times 10^{-12}$ L (N = 3), and the aqueous cell volume fraction is assumed to be 0.7. There was also the appearance of a higher concentration of GSHEt in $P388D_1$ cells, 17.84 nmol/10^6 cells or *ca.* 10.3 mM, and a low concentration of $GSHEt_2$, 1.04 nmol/10^6 cells or 0.60 mM. This is attributed to the entry of intact $GSHEt_2$ into the cytosol of cells and the subsequent hydrolysis of the ester groups of $GSHEt_2$ by cytosolic non-specific esterase. The presence of a low concentration of $GSHEt_2$ in the cytosol and a high concentration of GSHEt suggests that $GSHEt_2$ was hydrolysed rapidly to GSHEt, whereas GSHEt was hydrolysed slowly to GSH in $P388D_1$ cells. $GSHEt_2$ (10 mM) therefore was capable of effectively loading an extra 14.2 mM GSH derivatives into $P388D_1$ cells. GSH diester has been prepared and evaluated for the prevention of radiation-induced oxidative stress [43], and the loading of bovine endothelial cells [44], human blood cells, tumour cells, fibroblasts and lymphocytes with GSH derivatives [21]. In some reports [43, 44], the GSH diester was cytotoxic and caused vacuolization of cells. Similar effects were not observed herein or by others [21]. This discrepancy may be attributed to impurities in the GSH diester preparations [20].

There was also a marked increase in the cellular concentration of γ-glutamylcysteine in $P388D_1$ cells incubated with 10 mM $GSHEt_2$. This is surprising since high concentrations of GSH inhibit γ-glutamylcysteine synthetase activity [23] and this effect of $GSHEt_2$ had not been reported previously [21, 43, 44]. γ-Glutamylcysteine was probably formed by the γ-glutamylcysteine synthetase-independent mechanism involving the formation of γ-glutamylcystine in the extracellular medium from the degradation of reduced glutathione diester, delivery into cells and thiol-disulphide exchange to form γ-glutamylcysteine [24]. γ-Glutamylcysteine may then be converted to GSH, which is not feedback inhibited by GSH. $GSHEt_2$ is thereby able to stimulate *de novo* synthesis of GSH. Availability of cyst(e)ine is thought to be a rate-limiting precursor of GSH synthesis in cells [45]. The extracellular formation of γ-glutamylcystine and its delivery into cells is a novel and possibly important source of cyst(e)ine supply for GSH synthesis [38].

GSH, GSHEt and $GSHEt_2$ all degraded to non-thiol-containing products in the absence of cells when incubated in MGB at 37° for 3 hr, except $GSHEt_2$ also underwent spontaneous hydrolysis of the γ-glutamyl ester to form also GSHEt. GSH derivatives may oxidize to form the corresponding disulphide but also sulphonic acid GSO_2H and sulphonic acid GSO_3H derivatives [46] (*cf.* the autoxidation of cysteine [37]) but these were not characterized herein.

GSH diester is a potent vehicle for the delivery of GSH into cells. The apparent facile entry of the GSH diester into cells and rapid hydrolysis to GSH monoester, and slow subsequent hydrolysis of the monoester to GSH, has the effect of loading cells

with GSH monoester with slow release of GSH therefrom. This may be advantageous for prophylaxis where GSH monoester is deposited inside cells for slower release of GSH from the GSH monoester store. GSH monoester is a poor substrate for glutathione-dependent enzymes (glutathione reductase, glutathione transferase, glutathione peroxidase and glyoxalase I) but may prevent cytotoxicity by non-enzymatically reducing protein disulphides and scavenging reactive oxidizing and alkylating intermediates. The current study also suggests that GSH diester may also undergo extracellular hydrolysis by cell surface-associated esterase activity. Change of the ester group to decrease the reactivity towards extracellular esterases will enhance the bioavailability of GSH diester and enhance the antidote effect. The increase in the cellular concentrations of cysteine and γ-glutamylcysteine and the apparent stimulation of *de novo* synthesis of GSH by $GSHEt_2$ is an interesting development and deserves further investigation.

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