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Role of Cellular Thiol Status in Tocopheryl Hemisuccinate Cytoprotection against Ethyl Methanesulfonate-Induced Toxicity

Marc W. Fariss, Kay F. Bryson and Mark A. Tirmenstein*

Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, WA 99164-6510, U.S.A.

ABSTRACT. Suspensions of rat hepatocytes treated with the alkylating agent ethyl methanesulfonate (EMS) exhibited extensive lipid peroxidation as well as rapid and near complete depletion of cellular reduced glutathione (GSH) levels prior to cell death. Pretreatment of hepatocytes with medium deficient in sulfur amino acids accelerated cell death induced by EMS, confirming the previously reported cytoprotective role for GSH in this toxic event. Nearly all of the cellular GSH lost following 50 mM EMS treatment was accounted for as S-ethyl glutathione (GS-Et). No significant formation of glutathione disulfide was observed. The GS-Et formed was not exported from the cell but remained at high intracellular concentrations throughout the course of the experiment. In addition, EMS treatment inhibited the efflux of intracellular GSH and inhibited the cellular accumulation of glutamate (Glu). Supplementation of hepatocytes with 25 μM *d*-α-tocopheryl hemisuccinate (TS) protected these cells against EMS-induced lipid peroxidation and cell death. Cytoprotection with TS had no effect on EMS-induced depletion of intracellular GSH or intracellular levels of GS-Et or Glu. However, TS supplementation did prevent EMS-induced depletion of cellular protein thiols. Interestingly, the pretreatment of hepatocytes with 1 mM dithiothreitol promoted EMS toxicity. The results of this study suggest that the cytoprotective abilities of TS are related to the prevention of both EMS-induced lipid peroxidation and protein thiol depletion. Thus, the onset of lipid peroxidation and the loss of protein thiols in hepatocytes appear to be critical cellular events leading to EMS-induced cell death. BIOCHEM PHARMACOL 53;5:651–661, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ethyl methanesulfonate; tocopheryl hemisuccinate; glutathione; protein thiols; lipid peroxidation; hepatocytes

T† is an endogenous, lipophilic antioxidant that scavenges oxygen radicals and protects membranes against lipid peroxidation. The peroxidation of polyunsaturated fatty acids has been shown to disrupt cellular membranes and can lead to cell death. T is a normal constituent of most cellular membranes but can be consumed rapidly during periods of oxidative stress. Once T is consumed, cellular lipids are subject to peroxidation. Numerous *in vitro* studies have indicated that the supplementation of isolated hepatocytes with vitamin E can protect cells against lipid peroxidation. In this regard, studies conducted in our laboratory have shown that the vitamin E ester, TS, is superior to unesterified T in protecting hepatocytes against chemically induced lipid peroxidation and toxicity. Supplementation of isolated rat hepatocytes with TS protected against lipid peroxidation and cell death induced by cadmium [1], oxygen [2], the ionophore A23187, and EMS [3]. However, no protection was seen against the toxicity induced by these chemicals when hepatocytes were supplemented with T. In these studies, protection against lipid peroxidation correlated well with protection against cell death. The differences in protection with T and TS occurred despite higher cellular T levels following T rather than TS supplementation.

The mechanism of this enhanced protection by TS treatment remains unclear. One possible explanation is that TS is more water soluble than T and, as such, is more likely to be incorporated into cellular membranes in an "active" configuration when given *in vitro* [4, 5]. As a result, an enhanced antioxidant capacity from TS might promote the protection or regeneration of critical cellular functional components such as cellular thiols during a toxic insult. In this regard, EMS treatment is known to alkylate cellular

* Corresponding author: Marc W. Fariss, Ph.D., Department of Pharmaceutical Sciences, Washington State University, Pullman, WA 99164-6510. Tel. (509) 335-4689; FAX (509) 335-0162.

† *Abbreviations:* T, *d*-α-tocopherol; TA, α-tocopherol acetate; TS, *d*-α-tocopheryl hemisuccinate, or tocopherol succinate; GSH, reduced glutathione; GSSG, glutathione disulfide, GS-Et, S-ethyl glutathione; Glu, L-glutamic acid; γ-Glu-Glu, γ-glutamylglutamic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; TBA, thiobarbituric acid; PCA, perchloric acid; EMS, ethyl methanesulfonate; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; IAA, iodoacetic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; and DTT, *dl*-dithiothreitol.

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non-protein thiols such as GSH [6], and the resulting depletion of this sulfhydryl-containing compound has been proposed to play a role in EMS toxicity [7]. Thiol alkylation by EMS might also lead to the loss of cellular protein thiol groups. The decrease of protein thiol levels has been associated with the development of toxicity in isolated hepatocytes for numerous agents. Studies have suggested a link between the loss of protein thiols and the onset of cell death for such diverse chemicals as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [8], acetaminophen [9], *N*-acetyl-*p*-benzoquinone imine [10], menadione [11], 3-*tert*-butyl-4-hydroxyanisole [12], chloroacetaldehyde [13], ethacrynic acid [14] and Adriamycin [14], as well as many other toxic chemicals. Thus, the following study was conducted to examine more closely the effect of EMS on cellular protein and nonprotein thiol status and to determine whether TS protects hepatocytes from EMS toxicity by modulating cellular thiol status.

MATERIALS AND METHODS

Chemicals

Collagenase (Type IA), dibutyl phthalate, HEPES, LDH, methanesulfonic acid ethyl ester (EMS), β-NAD, PCA, EDTA, SDS, Trizma Base, Trizma–HCl, DTNB, thiobarbituric acid, MDA, IAA, *m*-cresol purple, FDNB, TS, γ-Glu-Glu, GSH, GSSG, GS-Et, Glu, DTT, and Triton X-100 were obtained from the Sigma Chemical Co., St. Louis, MO. Ingredients used to prepare Waymouth's medium were from either Sigma or Fisher Scientific (Pittsburgh, PA). Percoll was obtained from Pharmacia (Piscataway, NJ).

Preparation of Isolated Hepatocyte Suspensions

Adult male Sprague–Dawley rats weighing 125–150 g were obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN) and given food and water *ad lib.* in our animal facility for 1 week before use. Rat parenchymal liver cells were prepared by a collagenase perfusion method as described previously [15]. A yield of 5–6 × 10^8 cells was routinely obtained with greater than 85% viability. Hepatocyte suspensions (2 × 10^6 cells/mL, 20 mL) were prepared in 125-mL boiling flasks and slowly rotated at 37° under an atmosphere of 95% air/5% CO_2. Experiments were begun approximately 15 min after preparing hepatocyte suspensions by taking samples of cell suspension for analysis (see isolation of viable hepatocytes method and Fig. 1) at the 0 time point and then hourly thereafter. Following the 0 time point, cell suspensions were incubated with TS (25 μM, final concentration) or a vehicle control (15 μL EtOH or 0.075% final concentration) for approximately 15 min. Suspensions were then treated with EMS (35–50 mM, final concentration).

The medium was serum-free Waymouth MB 752/I, which was further modified by omission of GSH, hypoxanthine, phenol red, arginine, cysteine, and L-ascorbate. Additions to the medium (final concentration) included 0.67 mM methionine, 0.2 mM cystine, and 25 mM HEPES, and pH was adjusted to 7.4.

All animal procedures received prior approval by the University Laboratory Animal Care and Use Committee and met or exceeded current local and federal regulations.

Isolation of Viable from Nonviable Hepatocytes

At each time point, rapid separation of viable from nonviable hepatocytes and medium was accomplished by the dibutyl phthalate centrifugation method (see Fig. 1). Briefly, 0.5 mL of cell suspension was layered over a density gradient of dibutyl phthalate (d = 1.046) and 10% PCA (d = 1.06) or Percoll/Hanks' salt solution (d = 1.06). Following centrifugation (Eppendorf microcentrifuge; 13,000 *g* for 30 sec), biochemical analyses were conducted on the medium (the layer above dibutyl phthalate), the PCA cell extract, the lysed cell pellet (using PCA), and the intact cell pellet (using Percoll/Hanks' salt solution). By centrifuging hepatocytes through dibutyl phthalate, only viable cells (d = 1.07) are analyzed for alterations in cellular components. Nonviable cells (d = 1.02) remain above the dibutyl phthalate layer. Thus, only cellular events that occur dur-

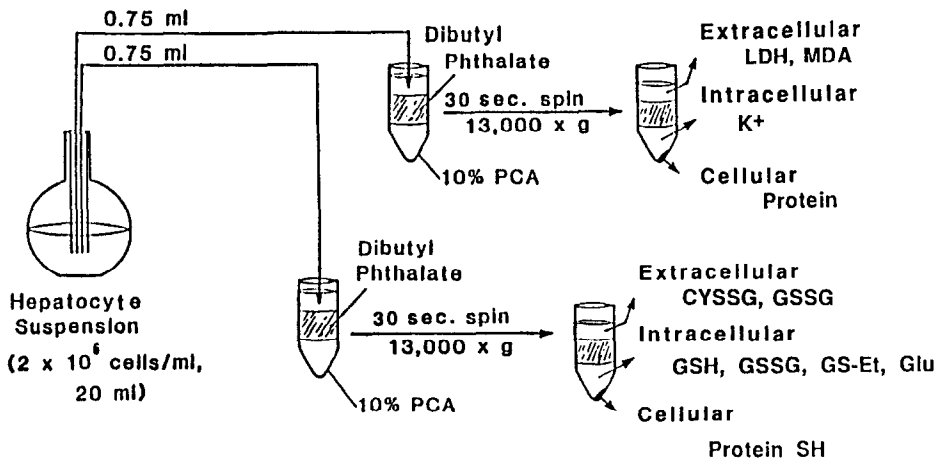


FIG. 1. Analysis of hepatocyte suspension for viability and intracellular and extracellular contents by the dibutyl phthalate separation method. Using this separation technique, only cellular events that occur during the toxic injury process and prior to cell death are measured in the cell pellet fraction (below the dibutyl phthalate layer).

ing the toxic injury process and prior to cell death are measured using this sampling technique [15].

In the present study, only viable hepatocytes were analyzed for cellular concentrations of K^+, GSH, GSSG, GS-Et, protein thiols and Glu. Cellular concentrations are expressed per 10^6 cells as determined by the protein content of the lysed cell pellet [16].

Cell Death

Cell death was determined by measuring the leakage of cellular LDH into the medium [15, 17] with a Cobas-Bio Clinical Analyzer (Roche Analytical Instruments Inc., Nutley, NJ). LDH quality control standards (Ciba Corning Diagnostics, Irvine, CA) were run simultaneously with each set of experimental samples. LDH was routinely measured in the medium (above the dibutyl phthalate layer).

Determination of Intracellular K^+

As a sensitive indicator of cell injury, the loss of intracellular [K^+] was measured as previously described [15]. Intracellular [K^+] was routinely determined in the 10% PCA lysate of viable cells (below the dibutyl phthalate layer) with a flame photometer (model No. 443, Instrumentation Laboratory, Watertown, MA). LiCl (0.1%) served as the internal standard, and a standard curve of K^+ was prepared with each experimental run.

Lipid Peroxidation

The extent of lipid peroxidation (MDA formation) in cell suspensions was determined by measuring the amount of TBA-reactive substances released into the medium [18] as described by the method of Stacey and Klaassen [19]. In brief, 0.25 mL of medium (above the dibutyl phthalate layer) was added to a tube containing 0.5 mL of 10% trichloroacetic acid and 0.05 mL of 2% butylated hydroxytoulene. Next, 1 mL of a 0.67% TBA solution was added to each tube, incubated at 90° for 20 min, cooled, and centrifuged, and the absorbance was read at 532 nm. Experimental values were extrapolated from an MDA standard curve (0.19 to 12.18 nmol) run with each experiment.

Intracellular Glutathione and Glutamate Measurements

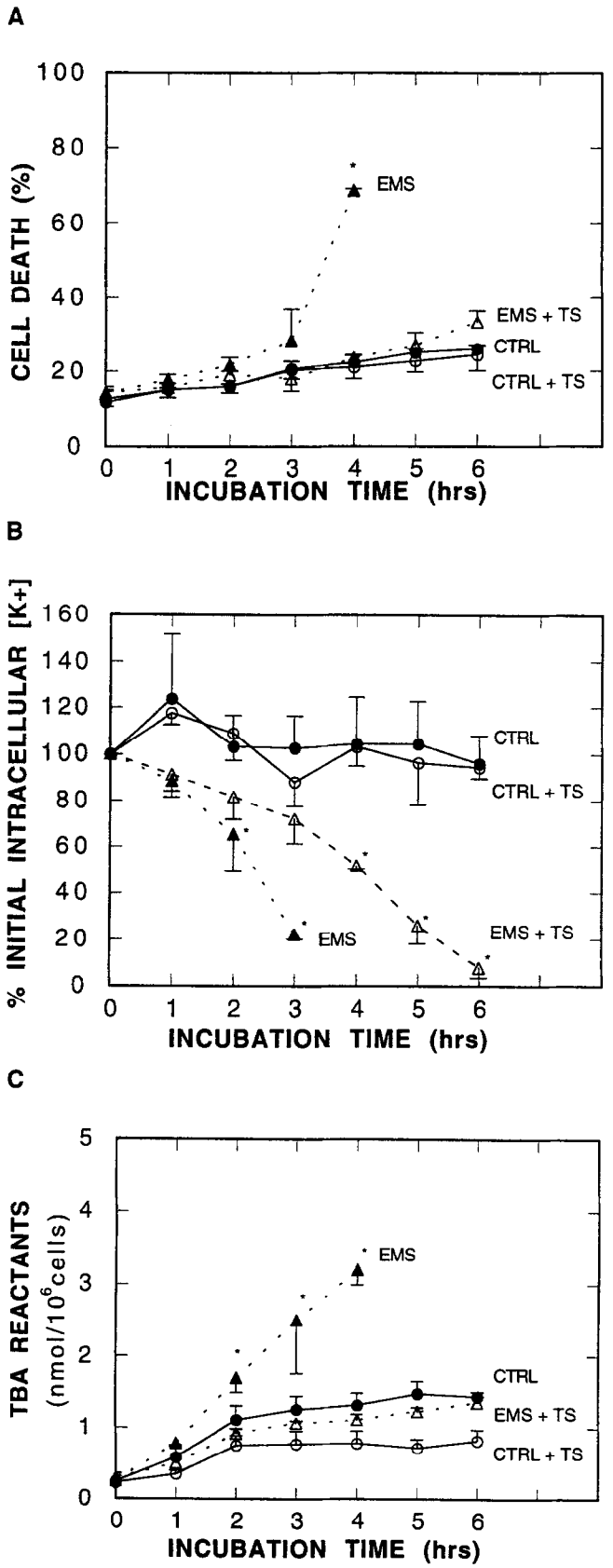
The intracellular concentrations of GSH, GSSG, GS-Et, and Glu were determined by the HPLC method of Reed *et al.* [20] as modified by Fariss and Reed [21]. In brief, a portion of the 10% PCA (containing a metal chelator, 1 mM bathophenanthrolinedisulfonic acid, from Aldrich, Milwaukee, WI) extract of viable cells (below the dibutyl phthalate layer, Fig. 1), was added to a tube containing 2 nmol γ-Glu-Glu (internal standard) and derivatized with IAA and FDNB. The resulting 2,4-dinitrophenyl derivatives of the *S*-carboxymethyl GSH and GSSG were separated and measured using a Hewlett Packard 1084 B HPLC equipped with a 3-aminopropyl column (5 mm, 20 cm, Custom LC, Houston, TX) and a Kratos UV detector (365 nm). After the derivatization, the solution was diluted 1:1 with 80% methanol, and 150 μL was injected onto the column; the mobile phase was maintained at 80% A (mobile phase A was 80% methanol) and 20% B (mobile phase B was 0.5 M sodium acetate in 64% methanol) for 5 min followed by a 10-min linear gradient to 1% A, 99% B at a flow rate of 1.5 mL/min. The mobile phase was held at 99% B until GSSG had eluted. The retention times for γ-Glu-Glu, GSH, and GSSG were approximately 19.2, 21.5, and 22.8 min, respectively. GSSG levels were corrected for 1% GSH oxidation that occurred during the assay procedure. When measuring Glu and GS-Et, the mobile phase gradient was altered to resolve these two derivatized compounds, using a mobile phase of 85% A, 15% B maintained for 20 min followed by a 1-min gradient to 1% A, 99% B (which was held until GSSG elution). The retention times for Glu, GS-Et, and GSH were approximately 11.0, 13.6, and 25.5 min, respectively. The limits of detection for Glu, GS-Et, and GSH were 1.0 nmol/10^6 cells and for GSSG, 0.5 nmol/10^6 cells. Standard curves for GSH, GSSG, GS-Et, and Glu were run with each experiment.

Extracellular Glutathione Measurements

A novel method described by Fariss and Reed [21, 22] for determining the selective efflux of GSH and GSSG from isolated hepatocytes was used. This method takes advantage of the thiol-disulfide interchange reaction between cystine (0.2 mM), which is added to the medium, and extracellular GSH resulting in stoichiometric formation of cysteinyl-glutathione disulfide (CySSG). Because the formation of the mixed disulfide does not affect extracellular GSSG, the cellular efflux of GSH and GSSG can be distinguished by analyzing cell medium for CySSG and GSSG concentrations, respectively, by the HPLC method described above. Using this method, the retention times for CySSG and GSSG are 19.8 and 22.8 min, respectively, and the limit of detection is 0.5 nmol/10^6 cells. A standard curve for each compound was run with each experiment.

Cellular Protein Thiol Measurements

The method of Di Monte *et al.* [23] was modified to measure protein sulfhydryl levels in viable hepatocytes with a Cobas-Bio Clinical Analyzer. The viable hepatocyte precipitate under the 10% PCA layer (see Fig. 1) was washed with 1 mL cold ethanol (to remove any residual GSH) and dissolved (sonicated) in 1.5 mL of 5% SDS with 0.02 M EDTA. An aliquot of this sample (200 μL) was placed in a Cobas cup and analyzed (in duplicate) for sulfhydryl content using 0.5 M Tris–HCl/5% SDS/0.02 M EDTA as buffer reagent No. 1 and 1.1 mM DTNB in methanol as buffer reagent No. 2. GSH standards were prepared in 5% SDS/0.02 M EDTA, and a standard curve was run with each experiment (assay was linear between 10 and 400 μM). The



Cobas instrument was programmed as follows: standard 1 concentration, 300 mmol/L; standard 2 concentration, 150 mmol/L; standard 3 concentration, 75 mmol/L; sample volume, 60 μL; diluent (buffer) volume, 30 μL; reagent (DTNB) volume, 50 μL; incubation temperature, 37°; incubation time, 10 sec; wavelength, 412 nm; time of first reading, 300 sec; time of reading interval, 10 sec; number of readings, 1. The Cobas instrument measures and records the change in absorbance (412 nm) per min.

Statistics

Data were analyzed for significance ($P < 0.05$) using single factor ANOVA and Scheffe's multiple comparison test (StatView II for Macintosh, version 1.04, Abacus Concepts Inc., Berkeley, CA).

RESULTS

Hepatocyte suspensions treated with 50 mM EMS demonstrated a time-dependent increase in cell death and lipid peroxidation and a time-dependent decrease in intracellular [K^+] (Fig. 2). Following a 3-hr incubation with EMS, hepatocytes showed a rapid and complete loss of cell viability over the next hour (Fig. 2A). EMS-induced cell death was preceded by a significant loss in intracellular [K^+] content (Fig. 2B) and a significant increase in lipid peroxidation (Fig. 2C). Supplementation of medium with 25 μM TS completely protected hepatocytes against lipid peroxidation and cell death induced by the EMS treatment. TS-treated cells also showed a significant delay in the loss of intracellular [K^+] induced by EMS exposure.

The protective effect of incubating hepatocytes with the GSH precursor methionine, prior to EMS treatment, is shown in Fig. 3. Hepatocytes were incubated in the presence and absence of 0.67 mM methionine for approximately 1 hr and then exposed to 50 mM EMS. Methionine pretreatment effectively delayed the expression of EMS-induced cell death. Control hepatocytes incubated with methionine for 1 hr had an intracellular GSH concentration of approximately 33 nmol/10^6 cells (Fig. 4A), while cells incubated for 1 hr in medium deficient in sulfur amino acids had an intracellular GSH concentration of approximately 20 nmol/10^6 cells (data not shown). These results confirm the findings of previous studies suggesting that intracellular GSH can indeed modulate the expression of EMS toxicity [7].

Control hepatocyte suspensions incubated in methio-

FIG. 2. Effect of vehicle (EtOH), TS (25 μM), EMS (50 mM), and TS (25 μM) + EMS (50 mM) on (A) cell death (LDH leakage), (B) the intracellular [K^+] of viable hepatocytes, and (C) lipid peroxidation in hepatocyte suspensions. Data points show the average of results obtained from three (± SD) separate hepatocyte preparations. Key: (*) significantly different from control cells at $P < 0.05$. The initial intracellular [K^+] was 1.2 ± 0.1 nmol/10^6 cells, and the medium contained 0.67 mM methionine and 0.2 mM cystine.

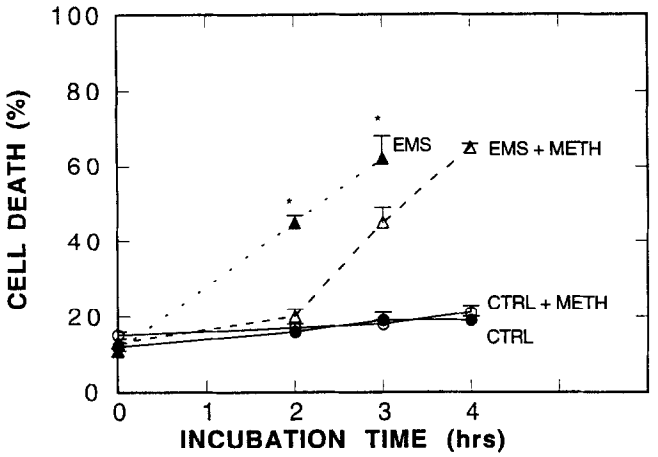


FIG. 3. Effect of GSH supplementation on EMS-induced cell death (LDH leakage). Hepatocyte suspensions were incubated with and without 0.67 mM methionine for approximately 1 hr prior to exposure to 50 mM EMS. Data points show the average of results obtained form three (± SD) separate hepatocyte preparations. Key: (*) significantly different from EMS + methionine-treated cells at $P < 0.05$.

nine-containing medium demonstrated a rapid increase in intracellular GSH levels during the first hour of incubation followed by maintenance of approximately 35 nmol GSH/10^6 cells for up to 6 hr (Fig. 4A). The addition of TS (25 μM) to the medium did not alter the intracellular GSH content of control cells during this 6-hr incubation period (Fig. 4A). In contrast, hepatocytes treated with 50 mM EMS exhibited a rapid depletion of cellular GSH (Fig. 4A). After 1 hr of incubation, intracellular GSH levels were reduced to approximately 15% of the initial value and remained severely depleted for the remainder of the experiment. A similar decline in intracellular GSH content was also observed in EMS-treated cells supplemented with 25 μM TS (Fig. 4A). The exposure of hepatocytes to EMS also resulted in the complete inhibition of the transport of intracellular GSH to the extracellular space (Fig. 4B and Table 1), and the complete inhibition of intracellular GSH formation (Table 1). In control cells during a 6-hr incubation, the rate of GSH efflux was constant at approximately 2 nmol/hr/10^6 cells (Fig. 4B and Table 1), and the rate of intracellular GSH formation was approximately 4 nmol/hr/10^6 cells. Treatment of cells with the cytoprotective agent TS did not alter significantly the effect of EMS or vehicle on GSH efflux or formation (Fig. 4B and Table 1). Rates of GSH formation were determined by measuring intracellular and extracellular levels of GSH and GSSG over time. The total amount of glutathione present, both intracellularly and extracellularly, was used to assess the amount of GSH synthesized. Intracellular and extracellular GSSG were not

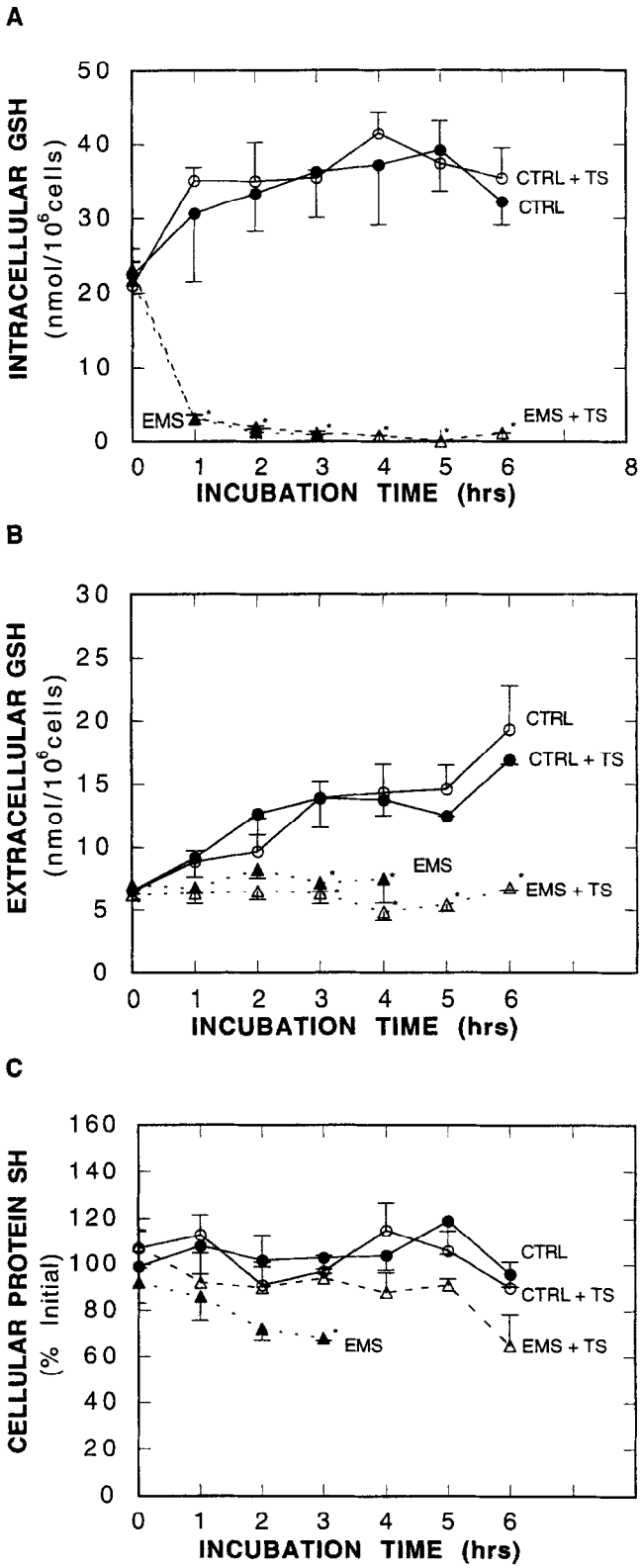


FIG. 4. Effect of vehicle (EtOH), TS (25 μM), EMS (50 mM), and TS (25 μM) + EMS (50 mM) on (A) intracellular GSH levels in viable hepatocytes, (B) extracellular GSH levels in hepatocyte suspensions, and (C) cellular protein thiol levels in viable hepatocytes. Data points show the averages of results obtained from three (± SD) separate hepatocyte preparations and are representative of two separate experiments. Key: (*) significantly different from control cells at $P < 0.05$. The initial intracellular GSH and cellular protein SH concentration values were 22.1 ± 2.2 and 124 ± 5 nmol/10^6 cells, respectively. The medium contained 0.67 mM methionine and 0.2 mM cystine.

TABLE 1. Effect of EMS and TS treatments on glutathione formation in hepatocyte suspensions

Glutathione formation	EMS	EMS + TS	Control	Control + TS
			(nmol/10^6 cells)	
Intracellular GSH	−22.1 ± 2.2	−20.9 ± 2.2	+9.8 ± 2.5	+15.4 ± 3.0
Extracellular GSH	−0.1 ± 0.4	−0.2 ± 0.9	+12.8 ± 2.8	+10.3 ± 0.3
Extracellular GSSG (2×)	ND	ND	+3.0 ± 0.3	+2.7 ± 0.1
Total glutathione formation	−22.2	−21.1	+25.6	+28.4
			(nmol/hr/10^6 cells)	
Rate of glutathione formation	ND	ND	4.3 ± 0.9	4.7 ± 0.8

Intracellular and extracellular glutathoine formations were determined by measuring glutathione pools at 0 time and 3 hr (EMS, EMS + TS), or 6 hr (control, control + TS) after treatment with vehicle (EtOH, control). TS (25 μM), EMS (50 mM) or EMS + TS. Total glutathione formation is the sum of the intracellular and extracellular glutathione pools. Values are means ± SD, N = 3. ND, not detected.

detected in hepatocyte suspensions during a 3-hr incubation with EMS (Table 1).

The putative alkylation product of EMS and GSH, GS-Et, was also measured in hepatocytes treated with EMS (Table 2). Intracellular levels of GS-Et increased to 18.0 nmol/10^6 cells after a 1-hr treatment with 50 mM EMS. Cellular GS-Et levels remained constant for the entire incubation period. At the 3-hr time point, cellular GS-Et levels (as well as GSH and Glu) were not detected due to increasing toxicity that severely limited the number of viable cells assayed. The formation of intracellular GS-Et closely reflected the loss of intracellular GSH in both a concentration and temporal manner. As shown in Table 2, hepatocytes incubated with EMS for 1 hr lost approximately 22 nmol GSH/10^6 cells and gained 18 nmol GS-Et/10^6 cells, which accounts for greater than 80% of the missing intracellular GSH. Since the coefficient of variation of these measurements is approximately 10%, the ethylation of GSH probably accounts for the GSH depletion observed. Cells supplemented with TS and exposed to EMS also exhibited intracellular GS-Et levels that corresponded to about 80% of the missing intracellular GSH. In EMS-treated hepatocytes, incubated in the presence and absence of TS, there apparently is a limited ability to export the alkylation product, GS-Et. GS-Et was not detected in vehicle control cells.

Cellular Glu levels were also affected by treatment with EMS (Table 2). Control cells exhibited a time-dependent increase in cellular Glu levels. Initial values rose from 7.8 to 24.2 nmol/10^6 cells after 1 hr. This increase in cellular Glu levels was not observed in cells treated with EMS regardless of TS supplementation. In fact, Glu levels continued to decline in EMS-treated cells throughout the course of the experiment, suggesting that EMS treatment inhibits the cellular accumulation of Glu.

Exposure to EMS also resulted in a decline in protein thiol levels in isolated hepatocytes (Fig. 4C). The dramatic decrease in intracellular GSH levels preceded by several hours the observed loss in protein thiols. Cellular protein thiol levels were reduced to about 65% of control levels following a 3-hr exposure to 50 mM EMS, while intracellular GSH levels were reduced to less than 15% of initial values after a 1-hr incubation with EMS. However, unlike the depletion of intracellular GSH levels, the loss of cellular protein thiols during EMS treatment was prevented by treatment with the cytoprotective agent TS. About 90% of the initial protein thiols were still present in TS-supplemented hepatocytes after a 5-hr exposure to 50 mM EMS.

The dithiol reagent DTT is used to convert disulfides to thiols and maintain sulfhydryl groups in the reduced state. Cells treated with 1 mM DTT for 15 min prior to 35 mM

TABLE 2. Effect of EMS and TS treatments on the intracellular concentration of GSH, GS-Et, and Glu in hepatocyte suspensions

	Intracellular concentration (nmol/10^6 cells)								
	GSH			GS-Et			Glu		
Incubation time (hr)	Vehicle control	EMS	EMS + TS	Vehicle control	EMS	EMS + TS	Vehicle control	EMS	EMS + TS
0	26.0 ± 2.0	28.2 ± 2.4	26.4 ± 4.6	ND	ND	ND	7.8 ± 4.0	8.8 ± 2.0	6.6 ± 1.0
1	27.4 ± 3.0	5.4 ± 1.2	4.2 ± 1.0	ND	18.0 ± 1.0	16.0 ± 2.4	24.2 ± 4.2	3.8 ± 0.6	3.6 ± 0.8
2	27.8 ± 2.2	4.2 ± 2.4	2.4 ± 0.4	ND	18.0 ± 1.4	17.6 ± 0.8	19.8 ± 2.8	2.0 ± 0.2	1.6 ± 0.2
3	23.6 ± 1.6	ND	1.6 ± 0.6	ND	ND	17.0 ± 1.8	14.8 ± 1.8	ND	1.6 ± 0.2

Hepatocyte suspensions were incubated with TS (25 μM) or vehicle control (EtOH), followed by EMS (50 mM) treatment. The concentrations of GSH, GS-Et, and Glu in viable hepatocytes were measured with an HPLC method, every hour during a 3-hr incubation. Values are means ± SD, N = 4. ND, not detected.

EMS exhibited a pronounced potentiation in both EMS-induced lipid peroxidation and cell death (Fig. 5). Almost 80% of the cells treated with the combined agents DTT and EMS were dead after 3 hr. For hepatocytes treated with 35 mM EMS alone, a 6-hr exposure period was required to produce a similar degree of cell death. DTT treatment alone did not increase lipid peroxidation or cell death over that observed in control cells.

DISCUSSION

Hepatocytes treated with EMS exhibited marked and rapid changes in cellular thiol status. EMS induced a rapid decline in intracellular GSH levels with 80–90% of cellular GSH being ethylated to form GS-Et. The near complete depletion of GSH occurred as early as 1 hr after hepatocytes were exposed to 50 mM EMS, and as such represented the earliest and most dramatic alteration in biochemical parameters measured in this study. GSH depletion preceded both EMS-induced lipid peroxidation and cell death. If hepatocytes were treated with medium void of methionine for 1 hr prior to the EMS treatment, the onset of EMS-induced toxicity was accelerated significantly. Since isolated hepatocytes supplemented with 0.67 mM methionine for 1 hr had approximately 13 nmol/10^6 cells more intracellular GSH than unsupplemented controls, these findings suggest a cytoprotective role for GSH in EMS toxicity, in agreement with previously published reports [6, 7]. However, supplementation of hepatocyte suspensions with 25 μM TS had no effect on the depletion of intracellular GSH by EMS, but TS was very effective in protecting against EMS-induced lipid peroxidation and cell death. Thus, the maintenance of intracellular GSH levels does not appear to be the mechanism by which TS provides cytoprotection. These results suggest that depletion of cellular GSH may be required for the onset of EMS-induced cell death but that GSH depletion alone is not sufficient to produce cell death. GSH depletion is likely to cause a series of cellular changes leading to the eventual death of the cell.

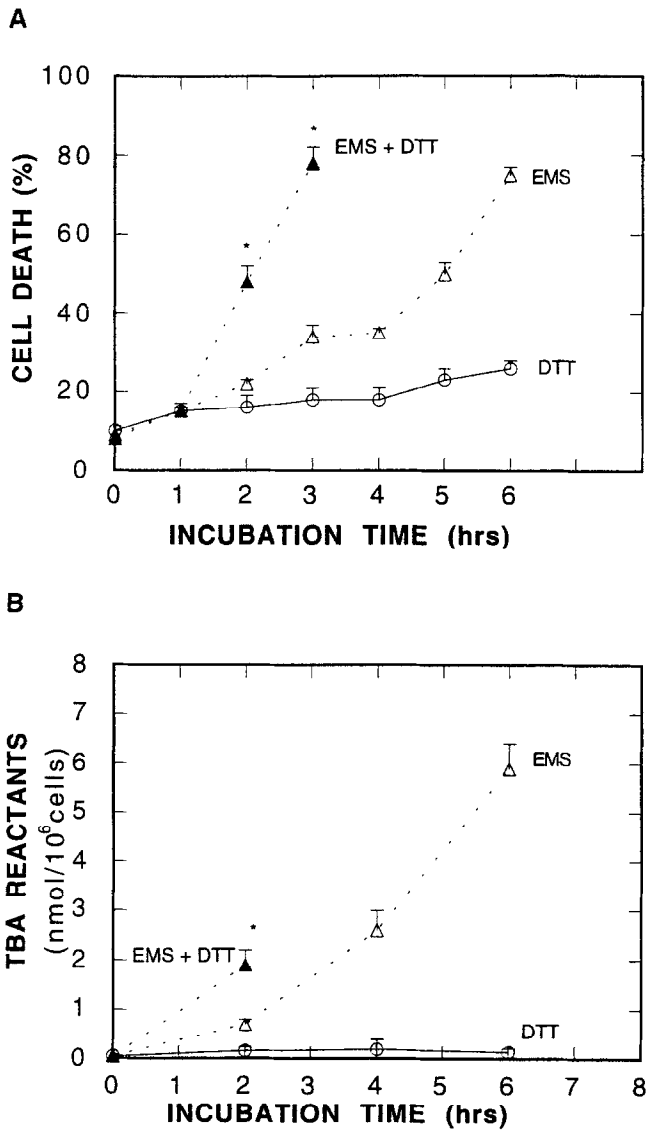


FIG. 5. Effect of DTT (1 mM) treatment on EMS (35 mM)-induced (A) cell death and (B) lipid peroxidation. Data points show the averages of results obtained from three (± SD) separate hepatocyte preparations. Key: (*) significantly different from EMS-treated cells at $P < 0.05$. Methionine and cystine were not present in the medium.

Another important effect of EMS treatment on hepatocyte function is the inhibition of cellular transport processes. The efflux of intracellular GSH and GS-Et was prevented by EMS as was the cellular accumulation of Glu. The mechanism for the adverse effect of EMS on cellular transport systems is unknown. However, the most likely explanations include the severe depletion of intracellular GSH content and the alkylation (inactivation) of critical thiols in the transport protein. The efflux of GSH from rat hepatocytes is a continuous process with approximately 2–5 nmol GSH/hr/10^6 cells secreted (Fig. 4B, Table 1), depending on the thiol/disulfide status of the medium [22, 24, 25]. Studies from Kaplowitz *et al.* [24] have demonstrated that the rate of GSH efflux from the liver is dependent on the hepatic concentration of GSH. Thus, due to the severe depletion of cellular GSH (>85%) in the present study, it seems reasonable that the reduced intracellular content of GSH is predominantly responsible for limiting the export of this nonprotein thiol. A second explanation, however, is that the alkylating agent EMS inactivated the sinusoidal GSH transporter by irreversibly ethylating critical protein thiols responsible for GSH transport function. Though this possibility is speculative, Lu *et al.* [25] have shown clearly that the rate of GSH efflux from hepatocytes is inhibited markedly under extracellular conditions that promote the loss of protein sulfhydryl groups.

It is now well known that parenchymal liver cells also contain an ATP-dependent GSH *S*-conjugate transporter (different from the GSH transporter) that is responsible for exporting GSH conjugates (and possibly other anionic compounds) from the cell [26]. Akerboom *et al.* [27] have

reported that the canalicular membrane transport of GSH conjugates is inhibited by 87% following incubation with the sulfhydryl reagent *N*-ethylmaleimide. Thus, the alkylation by EMS of critical protein thiols contained in the GSH *S*-conjugate transporter may also be responsible for the observed cellular retention of the glutathione conjugate, GS-Et. Additional studies are required to examine this possibility, and such an inhibition may also have important implications in cancer chemotherapy. Recent studies suggest that the GSH conjugate transporter may be the same or similar to the multidrug-resistance-associated (MDR) protein that can confer multidrug resistance to tumor cells by exporting drugs, thus lowering intracellular drug concentrations [28]. Such similarities encourage future studies on the effect of alkylating agents on MDR protein activity in order to develop strategies to prevent tumor cells from exporting cancer chemotherapeutic drugs.

Since the export of GSH conjugates is energy dependent and EMS treatment results in ATP depletion [29], another possible explanation for the inhibition of GS-Et efflux is the lack of cellular ATP. However the inhibition of GS-Et efflux appears to occur during the first hour of incubation with EMS when the maintenance of intracellular K^+ levels (ATP dependent [29]) are marginally affected. Thus, cellular energy status does not appear to be a factor in the observed inhibition of GS-Et export. Our experimental findings also suggest that the cellular accumulation of Glu is inhibited by EMS. In control cells, Glu levels were depleted following the isolation of hepatocytes (0 time) and, once placed in Waymouth's medium containing 1 mM Glu, dramatically increased (300%) over time. By contrast, EMS-treated cells demonstrated a continual decline in Glu levels over time. Because of the rapid and extensive increase in intracellular Glu levels of control cells during the first hour of incubation, we assume that the cellular uptake of Glu from the medium played a dominant role (as opposed to cellular Glu synthesis) in the observed accumulation [30]. Glu uptake by hepatocytes is a complex process that involves both Na^+-dependent and Na^+-independent anionic transporters [30]. However, the influence of intracellular GSH content or protein thiol status on the activity of the glutamate transport system has not been reported and, hence, requires additional study to delineate the mechanism of EMS-mediated inhibition. Because cytoprotective TS treatment did not alter the effect of EMS on GSH and GS-Et efflux and cellular Glu accumulation, the inhibition of cellular transport processes alone does not appear to be responsible for EMS-induced toxicity.

In addition to depleting intracellular GSH levels, EMS exposure also depleted cellular protein thiols and induced lipid peroxidation. However, in contrast to intracellular GSH, the toxic effect of EMS on protein thiols and membrane lipids was prevented by TS treatment. The mechanism by which EMS treatment induces lipid peroxidation in hepatocytes is unknown. EMS is a lipid-soluble alkylating (ethylating) agent that reacts by a combination of both S_N1 and S_N2 mechanisms [31]. Thus, the complete ionization (forming a carbonium ion and an anion) of the ethyl-oxygen bond of EMS does not occur prior to the reaction with an electron-rich center. As a result, the biological rate of reactivity of EMS is selective for thiol groups, being 50 times greater for sulfur compounds than nitrogen- and oxygen-containing compounds [31]. The predicted rapid alkylation of biological thiol groups by EMS agrees with the present study's observed rapid and near complete depletion of hepatocyte GSH levels and concomitant formation of GS-Et following EMS treatment. Since the reactivity of EMS and other esters of alkanesulfonic acids does not appear to involve free radical production (as suggested by the absence of GSSG formation and previous reports [31], our present findings implicate the loss of cellular antioxidants (such as GSH and T [3]) as a possible mechanism for EMS-induced lipid peroxidation. Once important cellular antioxidant systems are severely depleted, it seems likely that reactive oxygen species routinely generated by hepatocytes can now initiate lipid peroxidation [32]. This explanation is in agreement with the findings of other investigators who have shown that lipid peroxidation is associated with GSH depletion caused by the *in vitro* [32] and *in vivo* [33] administration of a variety of insults, including alkylating agents. In studies conducted with mice, bromobenzene, allyl alcohol, and diethyl maleate all severely depleted GSH, which led to the subsequent development of lipid peroxidation and liver necrosis [33]. In these same studies, animals fed a vitamin E-supplemented diet also showed severe GSH depletion but did not develop lipid peroxidation and were much more resistant to liver necrosis. Our study yielded similar results. TS supplementation was able to protect against lipid peroxidation and cell death in hepatocytes treated with EMS despite the near complete depletion of intracellular GSH. These results implicate lipid peroxidation as a critical cellular event in EMS-induced cell death and support the cellular release of the antioxidant T from TS as the mechanism for cytoprotection.

TS supplementation also protected hepatocytes against EMS-induced depletion of protein thiols. An important role for cellular protein thiols in maintaining cell viability during a toxic insult has been proposed by Orrenius [34] and many other investigators [8–14, 35, 36]. Previous studies have also demonstrated that cytoprotective TS treatment can prevent the loss of protein thiols in hepatocytes during a toxic insult with allyl alcohol, diethyl maleate, Adriamycin, and ethacrynic acid [14, 36, 37]. Though the mechanism responsible for this protection is unknown, the most likely explanation is that TS treatment increases membrane T and TS (reservoir of releasable T) content [1–3, 29], thus enhancing the antioxidant defenses of cellular membranes. In agreement with this conclusion is the work of Takenaka *et al.* [38] and Palamanda and Kehrer [39] demonstrating that membrane tocopherol is essential for preventing free radical-induced lipid peroxidation and the loss of protein thiols. Palamanda and Kehrer [39] also dem-

onstrated that the presence of GSH can prevent lipid peroxidation and spare both microsomal tocopherol and protein thiols during an oxidative challenge in rat liver microsomes. The studies of Takenaka *et al.* [38] showed that the oxidation of buried (intrinsic) protein thiols commenced only after tocopherol depletion and the induction of lipid peroxidation in red blood cell ghost membranes. These previous reports on the interrelationship between intracellular GSH and T content and the oxidation of membrane lipids and protein thiols are in agreement with the findings of the present study. Our findings demonstrate that intracellular GSH is more susceptible to depletion (alkylation) than protein thiols and that the loss of protein thiols occurs after the induction of lipid peroxidation. These results suggest that only following the loss of intracellular GSH and presumably T does lipid peroxidation occur, leading to the loss of protein thiols and cell viability. Based on the findings of previous investigators, we speculate that the observed loss of protein thiols in the present study might occur by oxidation directly from lipid peroxyl radicals and/or the lipid peroxidation product 4-hydroxynonenal [37] or by the exposure and subsequent oxidation or alkylation of intrinsic thiols due to the conformation change of the protein resulting from membrane oxidation [38]. Additional studies are required to investigate these potential molecular mechanisms for TS cytoprotection and EMS toxicity.

The addition of DTT to hepatocyte suspensions potentiated the toxicity of EMS, shortening the time required for both lipid peroxidation and cell death. This effect of DTT on chemical toxicity is unusual, with the majority of studies reporting a protective role for DTT pretreatment [9, 13]. DTT exposure may enhance EMS toxicity by several mechanisms. The reduction of certain protein disulfides or mixed disulfides by DTT may increase their susceptibility to irreversible alkylation or oxidation by EMS. Previous studies by Coan *et al.* [40] have demonstrated that the conversion of protein sulfhydryls to mixed disulfides can protect protein thiols against irreversible oxidation. These protein mixed disulfides can then be reduced back to thiols by a nonenzymatic GSH/mixed disulfide exchange reaction or by the cellular enzymes thioredoxin and glutaredoxin [41, 42]. However, in the present study, an increase in GSSG formation was not observed following EMS exposure, suggesting that EMS treatment does not form these potentially protective protein mixed disulfides. Thus, we speculate that the reduction of normal endogenous protein disulfides or mixed disulfides by DTT treatment may lead to irreversible alkylation (by EMS), resulting in protein conformation changes, the disruption of protein function, and the rapid loss of cell viability. Alternatively, Lu *et al.* [25] demonstrated that pretreatment of cultured isolated hepatocytes with 2 mM DTT for 30 min increased the efflux of GSH almost 5-fold and reduced intracellular GSH levels by about 35% after 30 min. Assuming a similar effect in the present study with a 15-min DTT pretreatment, lower intracellular GSH levels could make hepatocytes more vulnerable to the toxic effects of EMS. A final explanation is that low concentrations of DTT are known to react with iron and molecular oxygen to form reactive oxygen species and to promote liposome peroxidation [43]. DTT may also promote lipid peroxidation in EMS-treated hepatocytes, especially since cells are depleted of GSH and are very susceptible to any increased production of reactive oxygen species following treatment with EMS. Further experiments are required to determine which mechanism is responsible for the somewhat unique effects of DTT in EMS toxicity.

The findings from the present study suggest that the antioxidant properties of TS treatment are responsible for the observed cytoprotection against EMS-induced toxicity. Since the TS molecule itself has no known antioxidant properties, the cellular release of the antioxidant T from TS appears to be required for cytoprotection. This explanation is in agreement with our previous studies demonstrating that the inhibition of both TS hydrolysis and the resulting cellular accumulation of T (using esterase inhibitors [29] and non-hydrolyzable forms of TS [44]) eliminated TS cytoprotection against EMS-induced toxicity. In conflict with this explanation is our previous finding that hepatocytes treated with unesterified T or TA (25 μM) were not protected from the toxic effects of EMS (cell death or lipid peroxidation), despite a similar or greater increase in cellular T levels as compared with TS treatment [3, 29]. Other investigators have also reported the ineffective antioxidant and cytoprotective properties of T and TA added directly to *in vitro* systems [4, 45, 46]. It has been suggested that the extreme lipophilicity of T and its nonionic esters (TA) results (following *in vitro* administration) in T aggregates bound to the plasma membrane, which limits the antioxidant abilities of the measurable T and prevents the distribution of T to intracellular organelles and membranes [4, 5, 44–46]. By contrast, we and others speculate that by increasing the anionic nature or hydrophilicity of T (such as TS), the access, retention, and antioxidant abilities of T (in the form of TS and releasable T) in intracellular membranes are increased, thus providing cytoprotection [4, 29, 44, 46]. Unfortunately, the subcellular distribution of T and TS in hepatocytes treated with T or TS and its relationship to cytoprotection have not been examined adequately and represent an important area for future investigation.

Mitochondria are a subcellular site of particular interest in terms of defining the mechanism of EMS toxicity and TS cytoprotection. Our previous studies have demonstrated clearly that TS protects hepatocytes against EMS-induced loss of mitochondrial membrane potential and mitochondria structural changes [29]. In fact, numerous investigators have demonstrated that TS treatment protects cells and mitochondria against the toxic effects of a wide variety of mitochondrial toxicants including the calcium ionophore A23187 [47], extracellular calcium omission [48], Adriamycin [46] and oxygen [49]. Though the mechanism of mitochondrial protection by TS is unknown, Pascoe and Reed speculated that protection is related to the ability of TS to

rapidly enter hepatocytes and preferentially localize T (released from TS) in mitochondrial membranes. In agreement with this hypothesis, our data clearly show that TS is a mitochondrial protectant [29], prevents cellular lipid peroxidation and protein thiol loss, and is only protective following cellular hydrolysis to the antioxidant T [29, 44]. However, our present findings also indicate that TS treatment does not prevent EMS-mediated depletion of the cellular antioxidant GSH (which presumably includes the cytosolic and mitochondrial pools). Unfortunately, the effects of EMS and TS treatments on mitochondrial thiol status have not been investigated directly and represent exciting areas for future study. Since mitochondria are the main intracellular generators of reactive oxygen species, we speculate that the ability of TS to rapidly supplement these membranes with the antioxidant T provides sufficient antioxidant activity to prevent mitochondrial dysfunction and maintain cell viability during an insult with EMS.

In summary, by examining only viable hepatocytes for changes in cellular thiol status and toxicity during an insult with EMS, we observed the rapid and near complete loss of intracellular GSH content, the induction of lipid peroxidation, the inhibition of intracellular GSH and GS-Et efflux, and the inhibition of cellular Glu accumulation, prior to cell death. The ethylation of intracellular GSH by EMS appears to account for the observed loss of GSH. Several hours following the depletion of intracellular GSH and the induction of lipid peroxidation, but immediately prior to cell death, a significant loss of cellular protein thiols was observed only in viable hepatocytes exposed to EMS. The importance of these cellular perturbations in the toxic injury process was also investigated by examining these events in cells treated with the cytoprotective agent TS. In hepatocytes protected from EMS toxicity by TS treatment, only two of the toxic manifestations described above were prevented: the induction of lipid peroxidation and the loss of cellular protein thiols. Thus, we conclude that the cytoprotective abilities of TS are related to the prevention of both EMS-induced lipid peroxidation and protein thiol depletion and that these toxic changes appear to be critical cellular events leading to EMS-induced cell death.

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