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Research Article

The sesquiterpene lactone antineoplastic vernolepin acutely depletes murine tumor cell glutathione (GSH), and lyses the cells by an unknown mechanism that is enhanced synergistically by inhibition of GSH synthesis with buthionine sulfoximine (BSO) (Arrick et al. 1983. J. Clin. Invest. 71:258). We found here that lysis of P815 mastocytoma cells by vernolepin, with or without BSO, required cystine in the culture medium. Addition of catalase markedly suppressed vernolepin-mediated cytolysis in cystine-containing media, suggesting the involvement of hydrogen peroxide in the cytolytic action of vernolepin. Consistent with this, inhibition of tumor cell glutathione disulfide reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea or inhibition of endogenous catalase with aminotriazole synergistically augmented cytolysis by vernolepin. Moreover, H_2O_2 was released by suspensions of P815 cells in cystine-containing buffer (63 pmol/10(6) cells X h). Omission of cystine reduced the rate of H_2O_2 accumulation 10-fold. No H_2O_2 was detected without cells. Cytolysis by vernolepin could be restored in cystine-deficient medium by several other disulfides, themselves noncytolytic, such as disulfiram and oxidized Captopril, as well as by cysteine. In contrast, withholding two other essential amino acids (leucine or tryptophan) or adding cycloheximide did not interfere with cytolysis by vernolepin. These results suggest that cellular uptake of disulfides of physiologic and pharmacologic interest may be followed by their intracellular reduction and autooxidation with generation of H_2O_2. This previously unrecognized source of intracellular […]

Hydrogen Peroxide from Cellular Metabolism of Cystine

A Requirement for Lysis of Murine Tumor Cells by Vernolepin, a Glutathione-depleting Antineoplastic

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Abstract

The sesquiterpene lactone antineoplastic vernolepin acutely depletes murine tumor cell glutathione (GSH), and lyses the cells by an unknown mechanism that is enhanced synergistically by inhibition of GSH synthesis with buthionine sulfoximine (BSO) (Arrick et al. 1983. *J. Clin. Invest.* 71:258). We found here that lysis of P815 mastocytoma cells by vernolepin, with or without BSO, required cystine in the culture medium. Addition of catalase markedly suppressed vernolepin-mediated cytolysis in cystine-containing media, suggesting the involvement of hydrogen peroxide in the cytolytic action of vernolepin. Consistent with this, inhibition of tumor cell glutathione disulfide reductase with 1,3-bis(2-chloroethyl)-1-nitrosourea or inhibition of endogenous catalase with aminotriazole synergistically augmented cytolysis by vernolepin. Moreover, H_2O_2 was released by suspensions of P815 cells in cystine-containing buffer (63 pmol/10^6 cells · h). Omission of cystine reduced the rate of H_2O_2 accumulation 10-fold. No H_2O_2 was detected without cells. Cytolysis by vernolepin could be restored in cystine-deficient medium by several other disulfides, themselves non-cytolytic, such as disulfiram and oxidized Captopril, as well as by cysteine. In contrast, withholding two other essential amino acids (leucine or tryptophan) or adding cycloheximide did not interfere with cytolysis by vernolepin. These results suggest that cellular uptake of disulfides of physiologic and pharmacologic interest may be followed by their intracellular reduction and autooxidation with generation of H_2O_2. This previously unrecognized source of intracellular oxidant stress may be an important component of injury to GSH-depleted cells.

Introduction

Oxidant injury to cells is known to arise from a variety of exogenous agents, such as x-rays, drugs, or leukocytes (1–3). The role of glutathione (GSH)[1] in cellular defense against such injury is well established (4, 5). In contrast, toxicity to cells from oxidants of endogenous origin has rarely been noted (6). The evidence presented below implicates cystine metabolism as a previously unappreciated source of intracellular oxidant stress, one that can contribute to the death of GSH-depleted cells.

This work stemmed from our previous demonstration that the capacity of murine tumor cells to synthesize GSH is a critical element of their defense against antitumor compounds that rapidly deplete cellular GSH (7). In those studies, we used buthionine sulfoximine (BSO), a potent, nontoxic inhibitor of γ-glutamylcysteine synthetase (8, 9). Tumor cell lysis by jatrophone or the sesquiterpene lactones vernolepin, helenalin, elephantopin, or eriofertopin was markedly enhanced if the cells were exposed to BSO within 4–5 h of treatment with the antineoplastics (7). However, the mechanism of cytolysis by these agents remained unknown. In agreement with earlier studies on the role of the GSH redox cycle in defense of murine tumors against oxidant stress (9, 10), we also found that GSH-depleting antineoplastics sensitized tumor cells to lysis by H_2O_2 generated in the extracellular medium (7). The addition of BSO to sesquiterpene lactones markedly increased the degree of sensitization of tumor cells to H_2O_2 (7).

If the synergistic cytotoxicity of GSH-depleting agents and BSO were due solely to the inhibition by BSO of GSH synthesis, then it might be possible to substitute for BSO simply by withholding cystine, a source of cysteine essential for GSH synthesis in certain lymphomas (11–13). In the present report, we first demonstrated that P815 murine mastocytoma cells indeed depend on exogenous cystine for GSH synthesis. We next evaluated cytolysis of P815 cells resulting from a pulse with vernolepin followed by incubation in medium with or without cystine. Contrary to expectation, vernolepin-mediated cytolysis depended on cystine. Investigation into this phenomenon suggested that metabolism of cystine is a significant source of intracellular oxidant stress, which in turn appears to be a key element in lysis of tumor cells by vernolepin.

Methods

Streptomycin and penicillin were obtained from Flow Laboratories, Inc., McLean, VA. Horse serum was from Sterile Systems, Logan, UT. The following chemicals were from Sigma Chemical Co., St. Louis, MO: Triton X-100, dimethyl sulfoxide, 5-sulfosalicylic acid, NADPH, GSH, glutathione disulfide (GSSG), yeast GSSG reductase, superoxide dismutase, scopoletin, horseradish peroxidase, 3-amino-1,2,4-triazole, 5,5′-dithiobis-(2-nitrobenzoic acid), and disulfiram (tetraethylthiuram disulfide). Cycloheximide was from Calbiochem-Behring Corp., San Diego, CA, and catalase from both Sigma and Calbiochem. Dulbecco's phosphate-buffered saline, L-cystine, L-cysteine, ascorbate, and Eagle's minimum essential medium (MEM), provided as a kit in which the separate amino acids can be added as desired, were obtained from Grand Island Biological Co., Grand Island, NY; α-modified MEM specifically lacking cystine, cysteine, and ascorbate was prepared by

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1. *Abbreviations used in this paper:* BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; BSO, buthionine sulfoximine; GSH, glutathione; GSSG, glutathione disulfide; MEM, minimum essential medium.

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Grand Island Biological Co. Ascorbate content was restored at least 24 h prior to use. $Na_2{}^{51}CrO_4$ was from New England Nuclear, Boston, MA. DL-Buthionine-SR-sulfoximine was from Chemical Dynamics Corp., S. Plainfield, NJ. Vernolepin (NSC 106398) was obtained through the courtesy of Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, and Dr. J. D. Douros, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. From Bristol Laboratories we obtained 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, NSC 409962). Captopril (1-[(2S)-3-mercapto-2-methyl-1-oxopropyl]-L-proline) and its disulfide were gifts of E. R. Squibb and Sons, Inc.

Tumors. The P815 mastocytoma cell line was maintained by intraperitoneal passage in histocompatible mice as described (14). L1210 murine lymphoma and Daudi human lymphoma cell lines were a gift of I. Gresser. For experiments, tumors were grown in stationary suspension cultures in α-modified MEM, supplemented with 100 μg/ml of streptomycin, 100 U/ml of penicillin, and 10% heat-inactivated horse serum, except for L1210 and Daudi, for which fetal bovine serum was employed.

Use of pharmacologic agents. GSH depletion by BSO (0.2 mM) was achieved by incubation of cells in α-modified MEM, 10% horse serum at 37°C in 5% CO_2, 95% air. BSO stock solution (20–45 mM) was stored at 4°C for up to 3 wk before use. Vernolepin stock solutions (20–60 mg/ml in dimethyl sulfoxide) were stored at −70°C for up to 6 mo before use. Cells ($1.5–3 \times 10^6$/ml) were exposed to vernolepin in cystine-free MEM, 5% horse serum (with 0.1–0.5% dimethyl sulfoxide). Inhibition of GSSG reductase by BCNU was as follows: tumor cells (6.5×10^6/ml in cystine-free MEM, 5% horse serum) were incubated with BCNU or vehicle alone (final dimethyl sulfoxide concentration, 1%) at 37°C for 15 min, and then washed. BCNU was stored at −20°C and dissolved immediately before use. Where noted, 50 mM aminotriazole was present throughout the 18–20-h cytolysis assay period.

Biochemical assays. Total cellular glutathione (GSH plus GSSG) was determined as previously described (9, 15). Total glutathione is expressed as nanomoles of the tripeptide per milligram of cell protein. GSSG reductase was assayed by the method of Roos et al. (16) as described by Nathan et al. (10). GSH peroxidase activity was measured by the method of Paglia and Valentine (17). Catalase was measured according to the method of Baudhuin et al. (18). Protein content was determined by the method of Lowry et al. (19) using bovine serum albumin as standard. H_2O_2 production by tumor cells was measured by suspending 5×10^6 cells with viability ≥95% as determined by trypan blue exclusion in Krebs-Ringer phosphate buffer with 5 mM glucose (20), 2 μM scopoletin, and 1 mM NaN_3, with or without the following, as indicated in Fig. 4: one purpurogallin unit/ml horseradish peroxidase, 3000 Sigma units/ml catalase, and 90 μM cystine, in a total volume of 3 ml in a quartz cuvette thermostatted at 37°C in a Perkin-Elmer MPF4A fluorometer (Perkin-Elmer Corp., Norwalk, CT). The horseradish peroxidase-dependent oxidation of fluorescent scopoletin to a nonfluorescent product was recorded (20).

Cytolysis assays. Cytolysis experiments were performed either in cystine-free MEM or in α-modified MEM specifically lacking cystine and cysteine. It should be noted that incubation of P815 cells in cystine-free medium for up to 22 h was nontoxic, as judged by trypan blue exclusion or release of ^{51}Cr. P815 cells were first washed into cystine-free medium, 5% horse serum. Cells were labeled with $Na_2{}^{51}CrO_4$ either before (~50 μCi/ml for 30 min, then washed), or during (~10 μCi/ml for 1 h) incubation with vernolepin (cells at 1.5×10^6/ml). After vernolepin exposure, cells were washed (three centrifugations), and then incubated for 18–20 h in medium, 5% horse serum (1.5×10^5/ml in 0.5 ml) with or without cystine (0.09 mM), with or without BSO (0.2 mM). In addition, cysteine, catalase, superoxide dismutase, and other thiol compounds were sometimes present during this post-vernolepin incubation, as indicated in the text. At this time, cells were centrifuged and 0.2 ml of supernatant sampled for gamma counting. Reported data points represent the mean of duplicate or triplicate determinations, as indicated. Calculation of percent specific release was as suggested by Arrick et al. (7). In some experiments, the ^{51}Cr release assay was validated by comparison to the release of lactate dehydrogenase into the medium. Lactate dehydrogenase was assayed by following the oxidation of NADH (0.09 mM) in 0.1 M phosphate buffer at pH 7.5 upon the addition of pyruvate (0.76 mM).

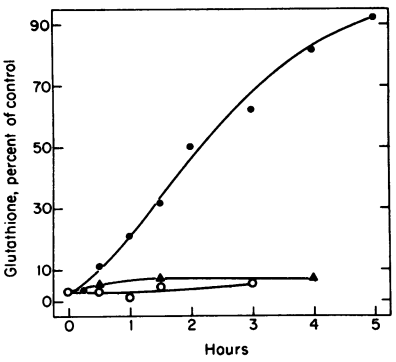

Figure 1. Effect of cystine on resynthesis of GSH after depletion by vernolepin: time course. P815 cells were incubated for 1 h in cystine-free MEM and 5% horse serum with 25 μg/ml of vernolepin, washed, and then incubated in the presence (●, ▲) or absence (○) of L-cystine (0.09 mM), in the presence (▲) or absence (●, ○) of BSO (0.2 mM). At the indicated times GSH content was determined. Data from two experiments are presented. Untreated cells contained 16.1±1.8 nmol/mg of protein.

Results

GSH synthesis in P815 cells depends upon exogenous cystine. To evaluate the dependence of GSH synthesis in P815 cells on the availability of cystine, medium specifically lacking in L-cystine was prepared (Eagle's MEM minus cystine). P815 cells were incubated with 25 μg/ml of vernolepin for 1 h in cystine-free medium, resulting in >95% depletion of GSH, washed, and then incubated with or without cystine. The time course of GSH resynthesis in the presence of cystine (0.09 mM) is illustrated in Fig. 1. GSH content was restored to 90% of control within 5 h. No significant GSH synthesis was observed in cystine-free medium or in medium containing cystine together with BSO. The dose-response of cystine-mediated GSH resynthesis is presented in Fig. 2. A log-linear

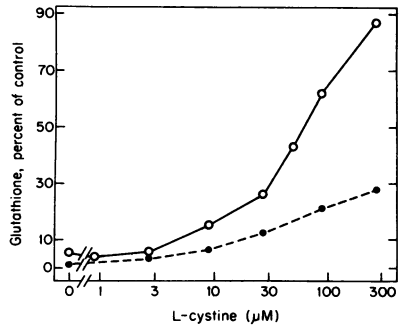

Figure 2. Effect of cystine on resynthesis of GSH after depletion by vernolepin: dose response. P815 cells were incubated for 1 h in cystine-free MEM and 5% horse serum with 25 μg/ml of vernolepin, washed, and then incubated with the indicated concentrations of L-cystine for 1 h (●) or 3 h (○), at which time GSH content was determined. Data from two experiments are presented. Untreated cells contained 16.1±1.8 nmol GSH/mg of protein.

relationship is evident for cystine between 9 and 270 μM. Cystine at 2.7 μM was insufficient to provide for detectable GSH synthesis by 3 h.

Effect of cystine on vernolepin-mediated lysis of P815 cells. With the knowledge that P815 cells require exogenous cystine for GSH synthesis, we expected that vernolepin-mediated lysis of cells in cystine-free medium would far exceed that obtained in cystine-containing medium, and thus would mimic that observed in complete medium in the presence of BSO. To our surprise, cytolysis, measured 18–20 h after a 1-h vernolepin pulse, was strongly dependent upon the presence of cystine. In one such experiment, illustrated in Fig. 3, P815 cells were pulsed for 1 h with 0, 50, or 250 μg/ml of vernolepin in cystine-free medium, washed, and then incubated for 18 h with various concentrations of cystine with or without BSO, at which time lysis was measured. There was no significant lysis in the absence of cystine. For cells pulsed with 250 μg/ml of vernolepin, significant lysis was observed with as little as 9 μM cystine with BSO present. Without BSO, equivalent lysis of these cells occurred with 90 μM cystine.

Verification of the ^{51}Cr-release assay as a measure of cell lysis under these conditions was obtained by parallel quantification of the release of lactate dehydrogenase from the cells into the medium. The data from one such experiment are presented in Table I.

Incubation of P815 cells in leucine- or tryptophan-free medium after the vernolepin pulse did not prevent cytolysis (Table II). Similarly, the addition of 25 μg/ml of cycloheximide to the post-vernolepin incubation in cystine-containing medium did not inhibit lysis. Thus, withholding other essential amino acids did not reproduce the effect of withholding cystine, nor did the cystine requirement of vernolepin-mediated cytolysis reflect a need for protein synthesis. However, as shown below, cysteine could support vernolepin-mediated lysis in place of cystine.

Exogenous catalase protects against cyst(e)ine-dependent cytolysis. Toxicity of exogenously added cysteine to cells in culture, inhibitable by catalase, has been reported (21–24). We wondered whether the toxicity to vernolepin-treated cells of exogenous cystine, serving as a source of intracellular cysteine, would be inhibited by catalase. As illustrated for one experiment in Table III, vernolepin-mediated cytolysis in the presence of either cysteine or cystine was partially inhibited by catalase, but not by superoxide dismutase. In three such experiments without BSO, in which 30–100 μg/ml vernolepin caused

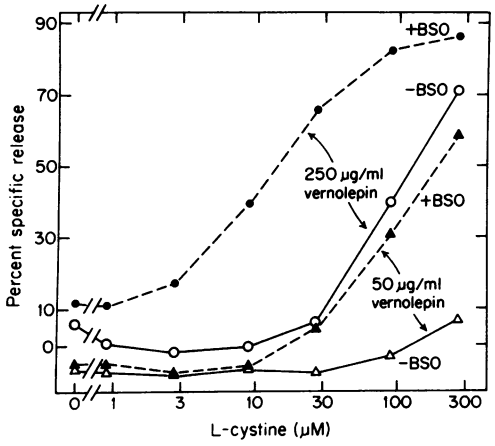

Figure 3. Effect of cystine on vernolepin-mediated lysis of P815 cells. P815 cells were pulsed for 1 h in cystine-free MEM and 5% horse serum with 50 μg/ml (△, ▲) or 250 μg/ml (○, ●) of vernolepin, washed, and then incubated for 18 h with the indicated concentrations of cystine, with (●, ▲) or without (○, △) BSO (0.2 mM), at which time ^{51}Cr release was determined. Points are means of triplicates. Standard error averaged 1%. Spontaneous release of ^{51}Cr after 18 h with the indicated concentrations of cystine from cells not exposed to venolepin was 18–20%.

Table I. Vernolepin-mediated, Cystine-dependent Lysis of P815 Cells: Comparison of Release of Lactate Dehydrogenase and ^{51}Cr

Incubation conditions		Vernolepin (1-h pulse)			
		50 μg/ml		200 μg/ml	
Cystine	BSO	LDH	^{51}Cr	LDH	^{51}Cr
μM	μM	% of specific release at 20 h			
0	0	6	−8	23	−3
0	200	10	−5	27	12
90	0	8	−6	21	8
90	200	36	30	73	73

P815 cells were incubated for 1 h with $Na_2{}^{51}CrO_4$ with or without vernolepin in cystine-free MEM with 5% horse serum, washed, and incubated for 20 h under the indicated conditions. Spontaneous release in medium with cystine was 7% for lactate dehydrogenase (LDH) and 21% for ^{51} Cr. Specific release (calculated as in reference 14) from cells not exposed to vernolepin was <2% for both markers. Enzyme assays are single determinations. ^{51}Cr release data are means of triplicates for which standard error of the mean averaged 1.5%.

Table II. Vernolepin-mediated Lysis of P815 Cells: Role of Exogenous Cystine, Leucine, or Tryptophan, and Effect of Cycloheximide

Incubation conditions		Vernolepin (1-h pulse)	
Complete medium	BSO	50 μg/ml	150 μg/ml
	μM	% of specific release at 20 h	
	0	3	22
	200	17	71
− Cystine	0	−4	0
	200	−2	4
− Leucine	0	1	31
	200	32	76
− Tryptophan	0	1	24
	200	24	78
+ Cycloheximide	0	0	28
	200	20	74

P815 cells were incubated for 1 h with $Na_2{}^{51}CrO_4$ with or without vernolepin in MEM lacking cystine, leucine, and tryptophane, with 5% horse serum, washed, and incubated for 20 h in the indicated media. In all conditions shown, cells not exposed to vernolepin had 0% specific release. Spontaneous release in complete medium was 24%. Data are means of duplicates for which standard error of the mean averaged 1.7%. Complete medium was MEM with 90 μM cystine, 400 μM leucine, 50 μM tryptophan, and 5% horse serum. Cycloheximide was 25 μg/ml.

Table III. Vernolepin-mediated Cyst(e)ine-dependent Lysis of P815 Cells: Effect of Exogenous Catalase and Superoxide Dismutase

Incubation conditions		Vernolepin (1-h pulse)	
MEM and additions	BSO	30 μg/ml	150 μg/ml
	μM	% of specific release at 18 h	
Cyst(e)ine-free	0	−9	0
	200	−2	7
+ Cysteine	0	−8	4
	200	18	73
+ Catalase	0	−11	−2
	200	−4	33
+ SOD	0	−11	6
	200	17	64
+ Cystine	0	−8	4
	200	14	69
+ Catalase	0	−6	0
	200	−3	37
+ SOD	0	−7	11
	200	19	68

P815 cells were incubated with $Na_2{}^{51}CrO_4$ with or without vernolepin in α-MEM lacking cysteine and cystine (with 5% horse serum), washed, and incubated for 18 h under the indicated conditions. There was no lysis of cells not exposed to vernolepin. Spontaneous release in medium without cysteine or cystine was 22%. Data are means of duplicates, for which SEM averaged 2.2%. Where indicated, cysteine was present at 530 μM, cystine at 90 μM, catalase at 2,000 Sigma U/ml, and superoxide dismutase (SOD) at 150 U/ml.

−1.3±6.5% (mean±SEM) lysis of P815 in cystine-free medium and 39.4±8.7% lysis in medium with cystine, 2,000 U/ml catalase inhibited lysis by 108±14%. In six experiments conducted in the presence of BSO, 30–100 μg/ml vernolepin led to −4.9±5.4% lysis without cystine and 59.8±11.0% lysis with cystine: the latter was inhibited 70.1±11.0% by catalase. Boiled catalase and equal weights of bovine serum albumin were without effect (not shown).

Effect of inhibition of endogenous GSSG reductase and catalase. The hypothesis that vernolepin-mediated cytolysis is due to the intracellular generation of H_2O_2 from the autoxidation of cysteine suggests that interference with tumor cell antioxidant pathways, beyond the GSH depletion brought about by vernolepin, might increase cytolysis even further. Inhibition of GSSG reductase with BCNU has been shown to augment H_2O_2-mediated lysis of P815 cells in vitro and in vivo (10, 25). Table IV presents data from two experiments in which P815 cells were treated with BCNU for 15 min followed by a 1-h incubation with vernolepin in cystine-free medium. Cells were then evaluated for GSSG reductase activity and lysis after a 20-h incubation with or without cystine. Cells exposed to BCNU alone (10–30 μg/ml) were not lysed. The combination of BCNU and vernolepin was especially potent, resulting in near-complete cytolysis at doses of vernolepin which were minimally toxic without BCNU. This synergistic cytolysis was dependent upon the presence of cystine. Cytolysis due to BCNU alone at substantially higher concentrations (≥100 μg/ml) was not dependent upon exogenous cystine (not shown). As previously reported (10, 25), BCNU was a potent inhibitor of P815 cell GSSG reductase. In addition, vernolepin (30–100 μg/ml for 1 h) resulted in ~50% inhibition of GSSG reductase activity, regardless of prior exposure to BCNU (Table IV). In contrast, exposure of P815 cells to vernolepin did not significantly alter the activity of GSH peroxidase or catalase (not shown).

Inhibition of endogenous P815 cell catalase with aminotriazole during the 18- to 20-h post-vernolepin incubation enhanced lysis in the presence of cystine and BSO by an average of 3.2±1.4-fold (mean±SEM for three experiments). Without prior vernolepin exposure, there was no cytolysis with aminotriazole under the same conditions.

Table IV. Effect of BCNU and Vernolepin on Glutathione Disulfide Reductase and Lysis of P815 Cells

	Pretreatment			Cystine 0 μM		Cystine 90 μM	
	BCNU	Vernolepin	GSSG reductase	BSO−	BSO+	BSO−	BSO+
	μg/ml	μg/ml	% of control	% of specific release at 20 h		% of specific release at 20 h	
Experiment 1	0	30	57	−8	2	2	58
	3	0	20	7	1	−5	2
	3	30	10	15	20	14	88
	30	0	9	18	7	5	3
	30	30	4	23	22	88	91
Experiment 2	0	5	ND	9	10	20	16
	0	50	ND	11	20	14	93
	10	0	24	4	7	3	1
	10	5	ND	13	12	100	90

P815 cells were incubated with BCNU or vehicle (1% dimethylsulfoxide) for 15 min, washed, then incubated for 1 h with vernolepin or vehicle (0.15–0.25% dimethylsulfoxide) before determination of GSSG reductase activity, which is given as a percentage of that in vehicle-treated cells (34 nmol NADPH/min · mg of protein in exp. 1 and 25 nmol/min · mg in exp. 2). For cytolysis data, cells were ^{51}Cr-labeled during exposure to vernolepin or vehicle, washed, and incubated for 20 h under the indicated conditions. Spontaneous ^{51}Cr release in medium with cystine was 22% in exp. 1 and 16% in exp. 2. Data are means of duplicates, for which standard error of the mean averaged 2.9%. Cytolysis of vehicle-treated cells was <7% in exp. 1 and <2% in exp 2. ND, not done.

Cystine-dependent cellular production of H_2O_2. We are not able to measure accurately intracellular H_2O_2 production. However, from the ability of extracellular catalase to inhibit vernolepin's cystine-dependent cytotoxicity, we reasoned that production of H_2O_2 by cellular metabolism of cystine may lead to release of a portion of the H_2O_2 into the extracellular medium, where it can be quantified. Therefore, we incubated tumor cells with or without cystine in the presence of scopoletin, a fluorochrome which undergoes stoichiometric oxidation by H_2O_2 in the presence of horseradish peroxidase, yielding a nonfluorescent product. As shown in Fig. 4 *A*, P815 cells released ~0.063 nmol $H_2O_2/10^6$ cells/h over the period between 0.5 and 3.0 h after the addition of 90 μM cystine. In the absence of cystine, H_2O_2 release over the same period was 10-fold lower (0.0064 nmol/10^6 cells/h). The five experiments summarized in Fig. 4 *B* illustrate that H_2O_2 release not only required cystine, but was completely dependent on cells. The loss of fluorescence of scopoletin under these conditions was due specifically to H_2O_2, because the fluorescence changes were dependent on the presence of horseradish peroxidase and were abolished by catalase (Fig. 4 *B*). Similar results were seen in one experiment each with L1210 and Daudi tumor cells (not shown).

Lysis of vernolepin-treated P815 cells in the presence of thiols and disulfides other than cyst(e)ine. Cysteine is not the only thiol that autoxidizes with the concomitant formation of H_2O_2 (26, 27). For instance, diethyldithiocarbamate induces the production of H_2O_2 when incubated with human erythrocytes (27). This may reflect extracellular oxidation to the disulfide, disulfiram. Disulfiram is taken up by erythrocytes and reduced to diethyldithiocarbamate (28). We asked whether the capacity to enter cells, undergo reduction to the free thiol, and then autoxidize to the disulfide with the concurrent generation of H_2O_2, as suggested in this report for cystine, might be exhibited by other thiols of medical interest. As shown in Table V, incubation of vernolepin-treated P815 cells in the presence of disulfiram caused cytolysis, which was inhibited by catalase. Likewise, both Captopril and its disulfide resulted in cytolysis of vernolepin-treated cells. Here too, exogenous catalase afforded protection (Table V). Similar results were seen with 2-mercaptoethanol and dithiothreitol (not shown).

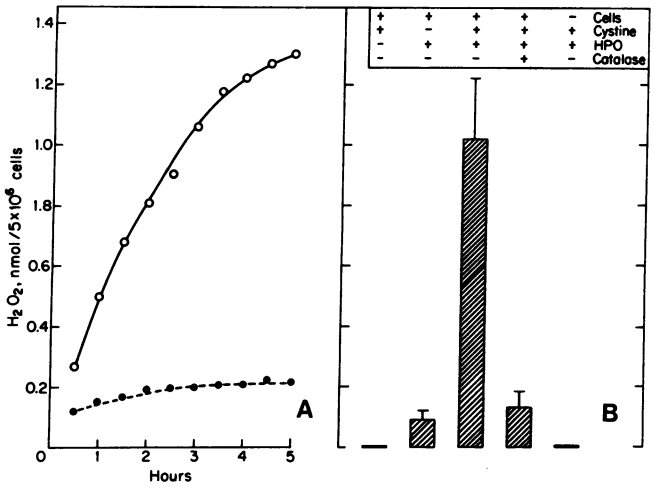

Figure 4. Cystine-dependent production of hydrogen peroxide by P815 cells. (*A*) Time course of H_2O_2 accumulation in the medium of 5×10^6 P815 cells incubated in Krebs-Ringer phosphate buffer, 5.5 mM glucose, with (○) or without (●) 0.09 mM L-cystine at 37°C, as detected by loss of fluorescence of scopoletin in the presence of horseradish peroxidase. (*B*) Results of 4–5-h incubations in five experiments like those in *A* (mean±SEM) with the deletions or additions indicated in the key. For concentrations of reagents, see Methods.

Discussion

In the course of studying the cytotoxicity of vernolepin, a GSH-depleting antineoplastic (7), we have obtained evidence implicating cystine metabolism as a source of intracellular oxidant stress. When antioxidant defenses are impaired, metabolism of cystine can contribute to cellular death. To our knowledge, an oxidant stress upon cells from their own metabolism of cystine has not previously been described. Nor has endogenous oxidant injury been recognized as an element in the mechanism of tumor cell lysis by sesquiterpene lactones or other sulfhydryl-reactive antineoplastics.

Murine P815 mastocytoma cells in tissue culture are dependent upon exogenous cystine for GSH synthesis, as reported for L1210 and L5178 lymphomas (12, 13). At least two additional murine lymphoma cell lines in tissue culture

Table V. Lysis of Vernolepin-treated P815 Cells in the Presence of Captopril and Disulfiram: Inhibition by Exogenous Catalase

Incubation conditions		Vernolepin (1-h pulse)	
MEM and additions	BSO	30 μg/ml	100 μg/ml
	μM	% of specific release at 18 h	
Experiment 1: cyst(e)ine-free	0	11	39
	200	17	52
+ Cystine	0	0	42
	200	85	89
+ Disulfiram	0	55	64
	200	64	74
+ Catalase	0	13	30
	200	17	37
Experiment 2: cyst(e)ine-free	0	3	16
	200	9	33
+ Cystine	0	8	40
	200	82	92
+ Captopril	0	29	41
	200	42	62
+ Catalase	0	−9	−7
	200	−8	−8
+ Captopril disulfide	0	0	61
	200	18	79
+ Catalase	0	−8	−6
	200	−7	−5

P815 cells were labeled with ^{51}Cr with or without vernolepin for 1 h in the cyst(e)ine-free medium (plus 5% horse serum) (α-modified MEM in Exp. 1, MEM in Exp. 2), washed, and incubated for 18 h under the indicated conditions. Spontaneous release of ^{51}Cr with cystine was 35% in Exp. 1 and 18% in Exp. 2. Specific release from cells not exposed to vernolepin was <10% for all incubation conditions. Where indicated, cystine was 90 μM, disulfiram 3 μM, catalase 2,000 Sigma U/ml, Captopril 100 μM, and Captopril disulfide 100 μM.

also required extracellular cystine for GSH resynthesis after depletion by vernolepin (TLX9 and P388, data not shown). In a series of lymphoid cell lines from leukemic and normal human subjects, Inglehart et al. (29) noted a correlation between cystine auxotrophy and leukemic origin: lymphoid cell lines from normals did not require cystine and contained detectable cystathionase activity, unlike cell lines of leukemic origin. Exploitation of this difference by cyst(e)ine depletion therapy has been suggested (30–32).

We have previously reported that BSO, a selective inhibitor of GSH synthesis, greatly augmented cytolysis by various sulfhydryl-reactive antineoplastic agents, such as vernolepin (7). We expected that P815 cells incubated in cystine-free medium would exhibit a level of susceptibility to lysis by vernolepin equivalent to that observed in the presence of BSO in medium containing cystine. Instead, cells in the absence of cystine appeared resistant to vernolepin-mediated lysis. Lysis in the presence of cystine was markedly enhanced by the addition of BSO after the vernolepin pulse. Incubation with vernolepin was in cystine-free medium, and thus cystine and vernolepin did not need to be present together for cytolysis to occur. In fact, addition of cystine as long as 8.5 h after the vernolepin pulse resulted in significant cytolysis measured 11.5 h later (not shown). Vernolepin-mediated cytolysis was not dependent upon two other essential amino acids (leucine or tryptophan) and was not inhibited by the addition of cycloheximide. Thus, a role for cyst(e)ine other than incorporation into either protein or GSH had to be sought to explain its action as a cofactor in cytolysis by vernolepin.

A number of investigators have described cysteine-associated toxicity, both in vivo and in vitro. The addition of cysteine to mammalian cell cultures was cytotoxic (22) in a manner inhibitable by catalase (21, 24). Carlsson et al. (23) found the bactericidal effect of cysteine under aerobic conditions to be enhanced by superoxide dismutase and abolished by catalase or metal ion-chelating agents. They further demonstrated metal-catalyzed production of H_2O_2 by cysteine, as have others

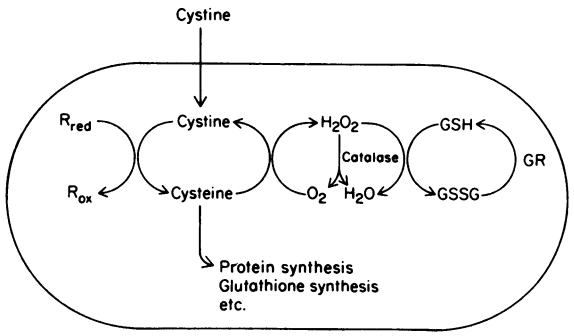

Figure 5. Cysteine autoxidation as a source of intracellular oxidant stress: a hypothesis. Cystine is taken into the cell and reduced by a factor (R_{red}) that may not be GSH. Most of the resultant cysteine is normally used for synthesis of GSH or protein or undergoes oxidation or transamination, but some autooxidizes, generating H_2O_2. The portion of cysteine that undergoes autooxidation may increase when protein synthesis and GSH synthesis are inhibited. Most of the H_2O_2 is normally catabolized by catalase, or by GSH peroxidase (not labeled). The reduction of H_2O_2 by GSH proceeds most efficiently when GSSG can be reduced back to GSH by GSSG reductase (GR). Depletion of GSH and inhibition of GR or catalase augment the toxicity of intracellularly generated H_2O_2.

(33). The generation of superoxide anion as a consequence of thiol autoxidation has been reported by Misra (34).

No H_2O_2 was generated by a cell-free solution of cystine, and extremely little by tumor cells in the absence of cystine. However, tumor cells plus cystine generated extracellularly detectable H_2O_2 at a rate that remained nearly constant for as long as the tumor cells were viable. However, the peroxide measured may markedly underestimate the amount formed, since H_2O_2 interacts with endogenous GSH and catalase in P815 cells (9, 10). Presumably we measured that portion of H_2O_2 that escaped these and other interactions. We speculate that the cells took up cystine and reduced it to cysteine, some of which autoxidized, generating H_2O_2. However, we have not directly demonstrated these postulated intracellular reactions of cyst(e)ine. It is possible that P815 cells produced H_2O_2 by another pathway in response to cystine.

It appears from these studies that the oxidant stress generated by metabolism of cystine is managed by a GSH-containing murine tumor cell, but can be lethal to the same cell if it is GSH-depleted in an appropriate manner. Depletion of GSH by prolonged incubation with BSO or by short-term exposure to 1-chloro-2,4-dinitrobenzene markedly enhanced susceptibility of P815 cells to oxidant injury (9). Moreover, vernolepin sensitized P815 cells to lysis by glucose oxidase-generated H_2O_2 (7). Thus, vernolepin-mediated GSH depletion might sensitize cells to the oxidant stress associated with intracellular cysteine oxidation. In support of this hypothesis, further interference with the GSH redox cycle by inhibition of GSSG reductase with BCNU dramatically enhanced vernolepin-mediated, cystine-dependent cytolysis. Inhibition of endogenous tumor cell catalase with aminotriazole had a similar though less marked effect. Finally, cytolysis was markedly inhibited by the addition of catalase to the incubation medium. Conceivably, this could reflect uptake of catalase by the cells, efflux of intracellular cysteine prior to autoxidation, or efflux of H_2O_2. The demonstration of H_2O_2 in the medium of cells incubated with cystine supports either of the latter two possibilities.

Our hypothesis is illustrated in Fig. 5. The mechanism of presumed intracellular reduction of cystine is unknown. Apparently, near-complete inhibition of GSSG reductase and depletion of GSH do not prevent cystine reduction. The results with other disulfides, such as disulfiram and oxidized Captopril, suggest the presence of a reductive pathway not specific for cystine.

GSH depletion by BSO alone does not result in cytolysis in cystine-containing medium. Vernolepin not only depletes GSH, but partially inhibits GSSG reductase. However, the combination of depletion of GSH by BSO and inhibition of GSSG reductase by BCNU was not cytolytic (not shown). This suggests that an additional effect of vernolepin is necessary for it to cause cytolysis in the presence of cystine, beyond the actions it shares with BSO and BCNU. Vernolepin may gain access to and deplete GSH in a compartment of the cell inaccessible to BSO. The incomplete penetration of BSO into murine hepatocyte mitochondria, with sparing of mitochondrial GSH, is consistent with this possibility (35). However, vernolepin has other effects on cells besides those considered here (e.g., 36–38), one or more of which may be required, together with oxidant injury, for its cytolytic action. For example, inhibition of protein synthesis by sesquiterpene lactones (37, 38), like inhibition of GSH synthesis by BSO (8), may block

a main route of utilization of intracellular cysteine, thereby increasing the pool available for autoxidation (Fig. 5).

Avoidance of oxidant stress from cysteine autooxidation may be a factor in the normal maintenance of remarkably low levels of intracellular cysteine. An analysis of the intra- and extracellular concentrations of free amino acids in HeLa cells grown in vitro has been provided by Piez and Eagle (39). Among essential amino acids, intracellular cysteine alone was maintained below the level of detection. The rapid mobilization of the cysteine within the GSH molecule for protein synthesis in rat liver identifies GSH as a storage form of cysteine (40), allowing the host quick access to cysteine without the need to maintain a potentially toxic intracellular pool. Cellular GSH can also serve to detoxify the H_2O_2 that may be generated by the cysteine that is present. Thus, as regards the potential for oxygen-dependent toxicity of intracellular cysteine, GSH metabolism may be of critical importance.

We have demonstrated that the toxicity of two additional disulfides, disulfiram and oxidized Captopril, is markedly enhanced by prior treatment of P815 cells with vernolepin. Cyclic oxidation-reduction of these thiols, as proposed above for cysteine, may be a source of intracellular H_2O_2. The relevance of such a mechanism to the inhibitory activity of disulfiram toward the malaria parasite *Plasmodium falciparum* (41) is suggested by the existence of other antimalarial drugs that generate an intraerythrocytic oxidant stress, notably primaquine (42). Similarly, autoxidation of homocysteine may underlie the damage to vascular endothelial cells, a cell population susceptible to oxidant injury (43, 44), which has been proposed to contribute to the development of homocystinemic atherosclerosis (45, 46).

These observations suggest that cyclic oxidation-reduction of thiols may play a role in pathogenesis, drug action, and drug toxicity.

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