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PROTECTIVE ROLE OF THE GLUTATHIONE REDOX CYCLE AGAINST ADRIAMYCIN-MEDIATED TOXICITY IN ISOLATED HEPATOCYTES

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Abstract—Incubation of isolated rat hepatocytes with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) resulted in the selective and extensive (> 90 per cent) inactivation of glutathione reductase. BCNU also depleted intracellular glutathione by 70 per cent but had no significant effect on cell viability or lipid peroxidation. Incubation of BCNU-treated hepatocytes with adriamycin (ADR) resulted in a decrease in cell viability concurrent with an increase in lipid peroxidation. These effects were not observed with untreated hepatocytes incubated with ADR. Glutathione depletion with diethylmaleate and incubation with ADR did not result in a significant decrease in cell viability or increase in lipid peroxidation. Incubation of BCNU-treated hepatocytes with ADR in the presence of exogenous α-tocopherol resulted in a significant amount of protection from ADR-mediated damage.

The antitumor benzanthraquinone adriamycin (ADR),† has been proven to be effective in the treatment of a broad spectrum of neoplastic diseases [1–3]. Its clinical use, however, has been complicated by a dose-limiting, cumulative cardiomyopathy [1, 4–6]. Lipid peroxidation has been reported to be associated with ADR cardiotoxicity [7]. Formation of an ADR semiquinone either spontaneously [8] or enzymatically, and its subsequent redox cycling to generate reactive oxygen species could explain the observed lipid peroxidation [9–13]. α-Tocopherol, a free radical scavenger, has been shown to reduce the cytotoxicity of ADR [7, 14, 15]. In addition, the depletion of intracellular glutathione levels has been considered as a factor contributing to ADR toxicity [15–17]. These findings suggest that ADR cytotoxicity may be the result of a free radical challenge that overwhelms the antioxidant protective systems of cells, including the glutathione redox cycle.

Several studies have demonstrated the protective nature of the glutathione redox cycle, which consists of glutathione in conjunction with glutathione reductase and at least two glutathione-dependent peroxidase activities [18–22]. Lipid peroxidation and cell damage could result from a failure to catabolize effectively hydroperoxides [23]. The glutathione redox cycle along with catalase and superoxide dismutase have been implicated in the catabolism of reactive oxygen species which, if unchecked, are capable of lipid peroxidative damage [19, 23–26].

The purpose of this study was to examine the toxicity of ADR from the standpoint of an oxidative challenge and to determine the extent of the protection afforded by the glutathione redox cycle, catalase, superoxide dismutase, and α-tocopherol during such an ADR challenge. We have used isolated hepatocytes as a model system to investigate, in particular, the role of glutathione reductase during drug-induced oxidative challenges. This was accomplished by selectively inactivating glutathione reductase with BCNU, thus allowing a direct assessment of the importance of this enzyme [27]. Additionally, the recent clinical use of ADR and BCNU in combination chemotherapy [28–30] heightened our interest in the possibility of synergistic effects of these drugs with respect to cytotoxicity.

MATERIALS AND METHODS

Hepatocytes isolation. Hepatocytes were isolated from fed, male Sprague–Dawley rats of 200–250 g body weight as described previously [31]. Both the preperfusion and perfusion solutions were buffered at pH 7.4 with 0.01 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes). The total length of the perfusion was less than 15 min. Aliquots of freshly isolated cells were immediately counted with a hemocytometer in 0.14% trypan blue solution containing 2% bovine serum albumin. Lactate dehydrogenase (LDH) leakage was used to determine cell viability. Cell suspensions with leakage of 9 per cent or less were used for incubations.

General conditions for hepatocyte incubations. Hepatocyte suspensions of 1.8×10^6 cells/ml were prepared in Fischer's medium [32] that was devoid of sulfur amino acids, contained 10% (v/v) sterile fetal calf serum, and was buffered at pH 7.4 with 0.01 M Hepes. The fetal calf serum added less than 1 nmole/ml of sulfur-containing amino acids to the medium. Incubations were performed in 25-ml Erlenmyer flasks under 95% O_2/5% CO_2 at 37° in a gyratory shaker. BCNU, DEM, and α-tocopherol

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† Abbreviations: ADR, adriamycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; LDH, lactate dehydrogenase; DEM, diethylmaleate; DMSO, dimethylsufoxide; GSH, reduced glutathione; GSSG, glutathione disulfide; and HMPS, hexose monophosphate shunt.

additions were made in DMSO as indicated in the appropriate figure legends. Adriamycin additions were made in saline. Controls with either DMSO or saline additions were also performed. Zero-time points were taken just prior to drug additions. At times indicated, 1.0-ml aliquots were removed for determination of cellular glutathione (GSH) levels, LDH leakage, trypan blue staining, lipid peroxidation and/or various enzyme activities. Controls indicated that DMSO additions had no effect on the indices examined. Data are presented as means ± standard deviation and in each case represent duplicate determinations from at least three separate preparations of hepatocytes.

Quantitation of intracellular glutathione levels. Hepatocytes and media, separated as described below, were analyzed for GSH by the high pressure liquid chromatography (h.p.l.c.) method of Reed *et al.* [33]. The method involves conversion of free thiols to *S*-carboxymethyl derivatives, reaction of amino groups with 1-fluoro-2,4-dinitrobenzene and separation of the resulting derivatives by reverse phase ion exchange h.p.l.c.

Determination of lipid peroxidation. Lipid peroxidation was monitored by measuring the formation of products that reacted with 2-thiobarbiturate as described by Fong *et al.* [34]. The data are presented as the change in absorbance at 532 nm with respect to zero-time absorbance.

Lactate dehydrogenase leakage. At the time indicated, two 0.5 ml-aliquots were removed. The medium was separated from cells in one aliquot by centrifugation (80 *g* for 3 min). The other aliquot was sonicated (Kontes sonicator at a power setting of 4) in the presence of 0.5% Triton X-100 for 30 sec and clarified by centrifugation. The supernatant fractions of both were then assayed individually by a modified procedure of Lindstrom *et al.* [35] with either a Beckman TR analyzer or a Cary 15 spectrophotometer.

Determination of other enzyme activities. Aliquots (1.0 ml) were removed just prior to drug addition (zero-time) and at times indicated after addition of drugs. Hepatocytes were either pelleted (80 *g* for 3 min), washed once with saline, resuspended in 0.5 to 1.0 ml saline, and sonicated with a Kontes sonicator at power setting 4 for 30 sec, or was allowed to settle and were sonicated directly in medium. Samples (5–50 μl) were then assayed for various enzyme activities as described below.

Glutathione reductase. Glutathione reductase activity was determined by monitoring the oxidation of NADPH at 340 nm in a Cary 15 spectrophotometer at 25° using the method of Worthington and Rosemeyer [36]. The assay mixture contained 0.2 M KCl, 1 mM EDTA, and 1 mM oxidized glutathione in 0.1 M potassium phosphate buffer, pH 7.0, and was initiated by the addition of NADPH to a final concentration of 0.1 mM.

Glutathione peroxidase. Enzyme activity was determined at 25° by a modified procedure of Paglia and Valentine [37]. Cell sonicate samples were added to assay solutions containing 0.3 mM NADPH, 3.8 mM NaN_3 (to inhibit catalase), 5 mM GSH, and 20 units yeast glutathione reductase. The reaction was initiated by the addition of H_2O_2 to a final concentration of 70 μM. The oxidation of NADPH was monitored at 340 nm.

Catalase. Catalase activity was determined by the method of Beers and Sizer [38] in which the reduction of hydrogen peroxide is followed spectrophotometrically at 240 nm at 25°. The change in absorbance that was inhibited by 3.8 mM NaN_3 was ascribed to catalase-mediated reduction.

Superoxide dismutase. Superoxide dismutase activity was determined by the inhibition of nitroblue tetrazolium reduction by superoxide according to the method of Winterbourn *et al.* [39].

Glutathione-S-transferase. Glutathione-*S*-transferase activity was determined by the method of Habig *et al.* [40] using 1-chloro-2,4-dinitrobenzene as the substrate.

Collagenase, Type IV for isolation of hepatocytes, was purchased from the Sigma Chemical Co., St. Louis, MO. High pressure liquid chromotography grade solvents were purchased from either Burdick & Jackson Laboratories Inc., Muskegon, MI, or the J. T. Baker Chemical Co., Phillipsburg, NJ. All reagents were reagents grade and commercially available. BCNU and ADR were supplied by the National Cancer Institute.

RESULTS

Our previous observations demonstrated that BCNU is a highly specific inactivator of glutathione reductase [41]. This stoichiometric inactivation of the enzyme *in vitro* led us to believe that BCNU might be employed to inactivate selectively the reductase in isolated hepatocytes. As Fig. 1 indicates, 75 μM BCNU inactivated more than 90 per cent of the hepatic glutathione reductase within 60 min. This level of inactivation was maintained throughout the

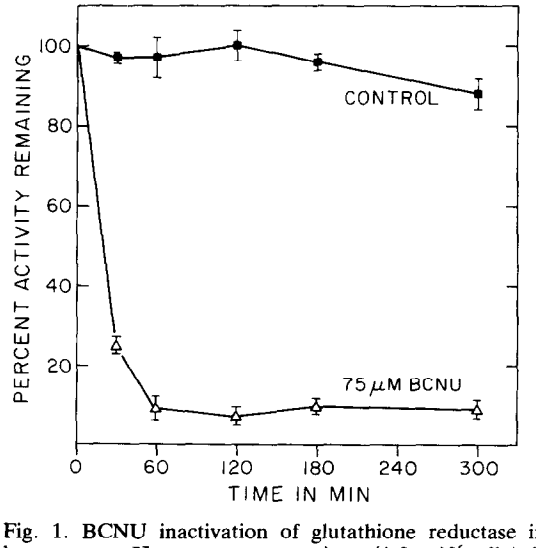

Fig. 1. BCNU inactivation of glutathione reductase in hepatocytes. Hepatocyte suspensions (1.8×10^6 cells/ml) were treated with either 0.01 ml of 95 mM BCNU in DMSO (△) or 0.01 ml DMSO (■) and incubated as described in Materials and Methods. The final concentration of BCNU was 75 μM. Immediately prior to drug addition (0 min) and at intervals shown above, 1.0-ml aliquots were removed and glutathione reductase activity was determined.

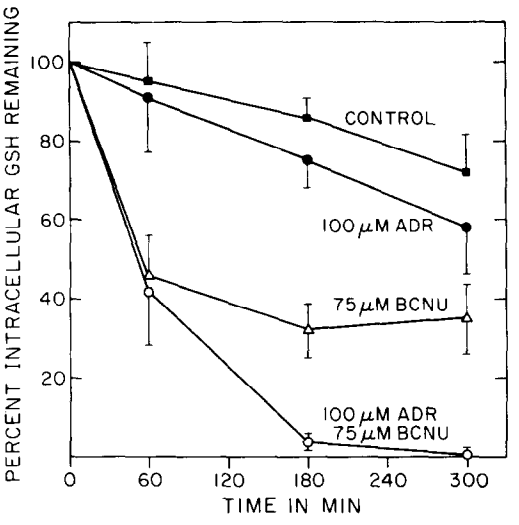

Fig. 2. GSH depletion in hepatocytes. Hepatocytes (1.8×10^6 cells/ml) were incubated either with 100 μM ADR in the presence of 75 μM BCNU (○) and absence of BCNU (●) or with 75 μM BCNU in the absence of ADR (△). Controls contained 0.1% (v/v) DMSO and 2.0% (v/v) saline, but no ADR or BCNU (■). Aliquots (1.0 ml) were removed and assayed for intracellular GSH. Incubation and assay conditions are described further in Materials and Methods.

remainder of the 5-hr incubation. BCNU (75 μM) had no significant effect on the hepatocytes as determined by either trypan blue staining, LDH leakage, or lipid peroxidation, the criteria used to assess oxidative damage. However, in the absence of supplemental sulfur-containing amino acids, the intracellular GSH level was decreased 70 per cent by BCNU when compared to the initial GSH level in control cells (Fig. 2). Thus, the inactivation proved selective

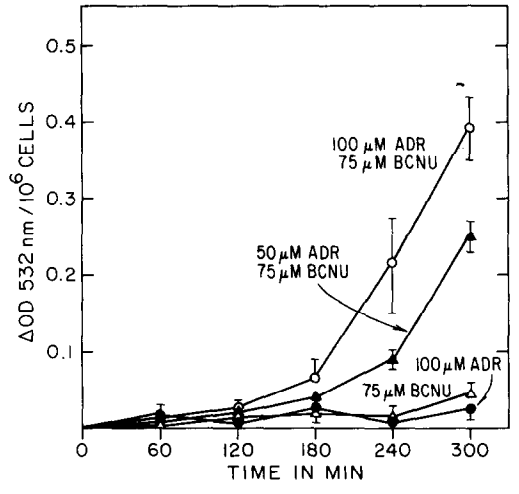

Fig. 3. Lipid peroxidation in hepatocytes. Hepatocytes were incubated with either 50 μM ADR plus 75 μM BCNU (▲), 100 μM ADR in the presence of 75 μM BCNU (○) and absence of BCNU (●), or 75 μM BCNU in the absence of ADR (△). Samples (1.0 ml) were removed and assayed for lipid peroxidation as described in the text.

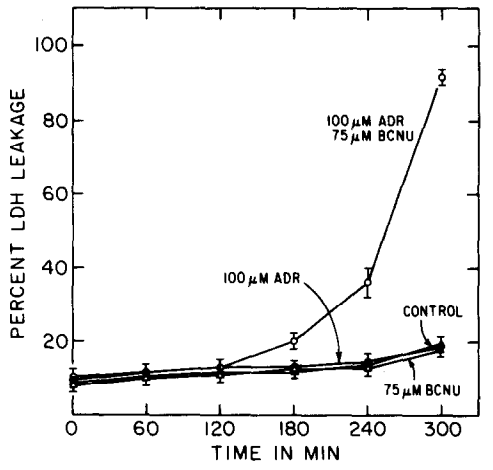

Fig. 4. ADR–BCNU-mediated lactate dehydrogenase leakage. Hepatocytes were incubated either with 100 μM ADR in the presence of 75 μM BCNU (○) and absence of BCNU (●), or with 75 μM BCNU in the absence of ADR (△). Controls contained 0.1% (v/v) DMSO and 2.0% (v/v) saline (▲). Cells were incubated and assayed for LDH leakage as described in the text.

enough to permit examination of the importance of the glutathione reductase and the glutathione redox cycle during an ADR-induced oxidative challenge.

ADR was used to challenge both normal and BCNU-treated hepatocytes. Fig. 2 illustrates the time-dependent effects of ADR and BCNU alone or in combination on the intracellular GSH levels of isolated hepatocytes. ADR (100 μM) only slightly reduced intracellular GSH levels when compared to corresponding control values. BCNU (75 μM) rapidly decreased GSH levels to 45 per cent of initial values within 60 min. The level of GSH decreased to and remained at 35 per cent of initial control values for the rest of the incubation. In combination, 75 μM BCNU and 100 μM ADR decreased GSH levels to the same extent as 75 μM BCNU alone by 60 min. However, the level dropped markedly to 5 per cent of initial values by 180 min with no GSH detectable at 300 min. Absence of methionine and cystine in the medium limited GSH synthesis and prevented recovery of GSH content in all of these experiments.

Figure 3 shows that the combination of 75 μM BCNU and ADR (50 or 100 μM) increased hepatocyte lipid peroxidation as monitored by the absorbance at 532 nm of 2-thiobarbiturate reaction products. The increase was found to be concentration-dependent with respect to ADR. Neither BCNU nor ADR alone caused any measurable increase in lipid peroxidation. Figure 4 indicates that BCNU and ADR in combination induced membrane damage as evidenced by the increased LDH leakage. This increase corresponded in time with the observed increase in lipid peroxidation (Fig. 3). Again, ADR (100 μM) or BCNU (75 μM) alone did not increase LDH leakage over that of control incubations.

To determine whether the increase in cell damage and lipid peroxidation was actually due to ADR, ADR at several concentrations (25, 50 and 100 μM) was incubated with hepatocytes in the presence of

Table 1. BCNU–ADR-mediated effects in hepatocytes

Drug			Cell viability*		Lipid peroxidation*
ADR (μM)	BCNU (75 μM)	α-Tocopherol (75 μM)	% LDH leakage†	% Trypan blue staining	ΔO.D. 532/10^6 Cells
Control	–	–	20 ± 2	22 ± 6	0.01 ± 0.004
–	+	–	22 ± 4	21 ± 5	0.039 ± 0.02
100	–	–	21 ± 4	22 ± 3	0.03 ± 0.01
25	+	–	27 ± 4	29 ± 9	0.085 ± 0.06
50	+	–	41 ± 8	48 ± 11	0.179 ± 0.08
100	–	–	84 ± 12	90 ± 10	0.499 ± 0.153
100	+	+	36 ± 11	42 ± 15	0.135 ± 0.09

* Lactate dehydrogenase (LDH) leakage at 0 time was 9 ± 2 per cent.
† Values were obtained after 5 hr of incubation.

75 μM BCNU. The increase in lipid peroxidation trypan blue staining, and LDH leakage proved to be concentration-dependent with respect to ADR (Table 1, Fig. 3). The percentage increases in both indicators of cell viability, LDH leakage and trypan blue staining, were essentially the same at each ADR concentration used. Values were approximately 28, 45 and 87 per cent with 25, 50 and 100 μM ADR respectively. The increase in lipid peroxidation corresponded well with the cell viability criteria. Both cell damage and lipid peroxidation due to 100 μM ADR plus 75 μM BCNU were decreased significantly by 75 μM α-tocopherol (Table 1).

An attempt was made to determine if the BCNU-mediated inactivation of glutathione reductase *per se* was primarily responsible for the increased hepatic sensitivity to ADR and not simply to the decreased intracellular GSH level. When 340 μM DEM was used to deplete intracellular GSH to 25 per cent of initial values at 180 min, no lipid peroxidation or cell damage was observed with either 50 μM or 100 μM ADR after 5 hr (Table 2). ADR (50 μM) in the presence of 75 μM BCNU, however, caused a similar decrease of GSH content (75 per cent) by 180 min, which was accompanied by significant lipid peroxidation and cellular damage. We wished to determine whether DEM could decrease ADR-mediated lipid peroxidation after glutathione reductase inactivation by BCNU. A combination of DEM and BCNU caused about the same degree of GSH depletion as the combination of BCNU and ADR but failed to cause a similar degree of LDH leakage or lipid peroxidation (Table 2). DEM did not prevent the extensive cellular damage caused by BCNU and ADR but instead increased both LDH leakage and lipid peroxidation (Table 2).

There was also the possibility that observed damage was the result of the inactivation of other enzymes with a role in the protective capacity of hepatocytes. To determine whether or not this was the case, the activities of enzymes known to afford cellular protection from oxidative challenges were measured. As Table 3 indicates, catalase, glutathione peroxidase, superoxide dismutase, and glutathione-*S*-transferase activities were not affected significantly by either ADR, BCNU or a combination of both.

DISCUSSION

A concentration-dependent decrease in cell viability, which was accompanied by increased lipid peroxidation, resulted from ADR treatment of BCNU-modified hepatocytes. Since BCNU selectively inactivated glutathione reductase, an important protective role of the glutathione redox cycle

Table 2. Effect of glutathione depletion on ADR-treated hepatocytes

Drug			%Intracellular GSH*	% LDH leakage	Lipid peroxidation (ΔO.D. 532 nm/10^6 cells)
			Time in min		
DEM (μM)	ADR (μM)	BCNU (μM)	180	300	300
Control			75 ± 3	20 ± 2	0.010 ± 0.006
340	0	0	32	20 ± 1	0.020 ± 0.006
340	0	75	4 ± 1	48 ± 10	0.188 ± 0.090
340	50	0	24 ± 6	19 ± 2	0.010 ± 0.002
340	100	0	28 ± 3	18 ± 2	0.040 ± 0.010
0	50	75	25 ± 1	47 ± 3	0.140 ± 0.020
0	100	75	10 ± 4	85 ± 16	0.538 ± 0.155
340	100	75	<1	92 ± 4	0.702 ± 0.122

* GSH content is expressed as percentage of zero-time control values.

Table 3. Effect of ADR-BCNU on certain hepatic enzyme activities

	Enzyme activity/10^6 cells*				
	Glutathione reductase	Glutathione peroxidase	Glutathione-*S*-transferase	Catalase	Superoxide dismutase
Zero-time	0.054 ± 0.010	1.00 ± 0.10	648.6 ± 68.3	24,556 ± 2,039	22.8 ± 2.6
180 min					
Control	0.058 ± 0.010	1.07 ± 0.08	614.5 ± 82.0	20,416 ± 1,726	19.3 ± 4.3
ADR + BCNU†	0.005 ± 0.001	0.92 ± 0.08	539.0 ± 48.0	21,052 ± 3,926	18.8 ± 3.5

* Activities are given in enzyme units as described in the text.
† Incubations contained 100 μM ADR and 75 μM BCNU.

is suggested by our results. This cycle, which utilizes glutathione peroxidase and glutathione reductase, provides reducing equivalents for H_2O_2 and organic peroxide detoxification [18].

The molecular mechanism of ADR toxicity has drawn much interest over the past several years. A view which is becoming more widely accepted is concerned with the ability of ADR to generate reactive oxygen species, thus causing cell damage. Pietronigro *et al.* [8] have demonstrated the spontaneous pH-dependent formation of ADR semiquinone radicals. This route of radical formation could be expected to occur only to a limited extent at physiological pH. A more likely route of semiquinone radical formation *in vivo* has been suggested by Handa and Sato [9, 10] and confirmed by Bachur *et al.* [11]. The latter group demonstrated the involvement of a univalent reduction of ADR by purified NADPH-dependent cytochrome P-450 reductase. Once formed, the semiquinone radical is then capable of reducing molecular oxygen, thus generating toxic oxygen species. This redox-cycling of certain quinones has been suggested by several groups as the probable cause of quinone-mediated oxidative damage to cells [9–13, 42]. The free radical nature of ADR toxicity is further supported by our results from experiments with BCNU-treated hepatocytes. ADR in combination with BCNU caused concentration-dependent increase in cellular damage which was accompanied by lipid peroxidation.

Lipid peroxidation has been implicated in membrane damage during oxidative stress that may lead to cell lysis [23, 43]. Enzymic protection against the initial reactive species of oxygen generated during such stress involves superoxide dismutase [44]. The rate of elimination of H_2O_2 and organic peroxide in ADR-treated hepatocytes in the absence of BCNU appeared adequate since cell viability was not affected (Table 1). Specific inactivation of glutathione reductase by BCNU treatment as shown here and in previous studies [17, 41, 45] caused loss of cell viability and extensive lipid peroxidation. We conclude that glutathione reductase acts in concert with glutathione peroxidase activity to provide a glutathione redox cycle which is vital to cellular defense against H_2O_2 and lipid peroxides. Exogenous α-tocopherol, a known free radical scavenger [7, 12, 13], greatly inhibited ADR damage in BCNU-treated hepatocytes (Table 1). Loos *et al.* [46] have shown that lack of glutathione reductase acivity renders human erythrocytes susceptible to drug-induced oxidative challenge. We have demonstrated previously a positive correlation between the abilities of several nitrosoureas to inactivate glutathione reductase and their myelotoxicity [41]. Sagone and Burton [17] observed an increase in the oxidation of [^{14}C-1]glucose and [^{14}C]formate upon administration of ADR to BCNU-treated and untreated glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes when compared to that of control erythrocytes. Control erythrocytes showed increased [^{14}C-1]glucose oxidation only after BCNU treatment. The oxidation of [^{14}C-1]glucose and [^{14}C]formate was taken as a measure of hexose monophosphate shunt (HMPS) activity and H_2O_2 generation respectively. They concluded that BCNU impaired the ability of erythrocytes to reduce ADR-generated H_2O_2 and this impairment was similar to that in ADR-treated G6PD-deficient erythrocytes. Although they concluded that glutathione peroxidase had a more important protective role than catalase, no attempt was made to assess the protective role of the glutathione redox cycle with respect to peroxidative damage.

The importance of heart tissue glutathione peroxidase in the detoxification of reactive oxygen species, which are generated via adriamycin, has been demonstrated in selenium-deficient mice [47]. Again, a major functional role of the glutathione redox cycle was postulated which supports our observations. None of the other protective enzyme activities we examined—glutathione peroxidase, glutathione-*S*-transferase, catalase and superoxide dismutase—were affected significantly by ADR or BCNU. Only glutathione reductase was inactivated significantly (> 90 per cent) by BCNU but not by ADR. We conclude that glutathione reductase as a component of the glutathione redox cycle has a major role in cellular protection from ADR-induced peroxidative damage in a coordinated response with superoxide dismutase, α-tocopherol, and related free radical scavengers.

Frischer and Ahmad [45], Sagone and Burton [17], and we, ourselves, have observed BCNU-related depletion of cellular glutathione. Is the ADR-induced damage occurring only after glutathione depletion and is the inactivation of the reductase required? When hepatic GSH levels were lowered by DEM to levels seen in BCNU-treated hepatocytes (25 per cent of control), ADR caused no observable oxidative damage (Table 2). Thus, at very low GSH levels, normal levels of glutathione reductase activity

appear to recycle effectively an equivalent low level of GSSG which is formed by the glutathione peroxidase reduction of H_2O_2 and organic peroxides. DEM treatment of hepatocytes did not prevent ADR-mediated damage after glutathione reductase inactivation (Table 2). This evidence showed that DEM did not interfere with cytochrome P-450 reductase participation in ADR-mediated generation of reactive oxygen species [9–13].

Although it is imprudent to extrapolate directly to clinical situations, our results do raise questions of clinical importance. For example, do biochemical defects or differences in tissues of various organs which impair the glutathione redox cycle indicate an increased sensitivity toward ADR and other oxidative drugs? Interestingly, the heart tissue of mice that exhibited ADR-induced cardiotoxicity contained relatively low levels of superoxide dismutase, catalase, and glutathione peroxidase activity [15, 47]. Certainly the results of Sagone and Burton [17], and our own, should be considered during combination chemotherapy involving both ADR and BCNU.

Our results demonstrate a major protective role for glutathione reductase as a participant in the glutathione redox cycle during an ADR-induced oxidative challenge. In addition, BCNU-treated hepatocytes appear to provide a promising model system with which to study the mechanism of drug-induced oxidative challenge.

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