Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 415-421

EFFECT OF BCNU PRETREATMENT ON DIQUAT-INDUCED OXIDANT STRESS AND HEPATOTOXICITY

Charles V. Smith

Section on Clinical Pharmacology, Department of Internal Medicine,
Baylor College of Medicine, Houston, TX 77030

Received January 21, 1987

Diquat administration produces hepatic necrosis in male Fischer-344 rats, and minimally in male Sprague-Dawley rats, with massive oxidant stress observable in both strains as evidenced by increased biliary efflux of glutathione disulfide (GSSG). Pretreatment of both strains of rats with 80 mg/kg of 1,3-bis(2-chloroethyl)-N-nitrosourea (BCNU) inhibited hepatic glutathione reductase by 75 percent and increased dramatically the biliary efflux of GSSG produced by administration of diquat. BCNU pretreatment markedly potentiated diquat hepatotoxicity in the Fischer rats and modestly in Sprague-Dawley rats. BCNU-pretreated Fischer rats did not show an enhanced depletion of nonprotein sulfhydryls in response to diquat, in spite of the dramatic potentiation of the hepatic necrosis produced, nor were protein thiols depleted. The effects of BCNU on diquat hepatotoxicity in the Fischer rat are consistent with a critical role for reactive oxygen species in the pathogenesis of the observed hepatic necrosis and for the protective role of the glutathione peroxidase/reductase system. The data suggest that shifts in thiol-disulfide equilibria are not responsible for the cell death produced by oxidant stress *in vivo*, but are consistent with a role for lipid peroxidation in the pathogenesis of the lesion.

The mechanistic involvement of reactive oxygen species has been proposed for tissue damage initiated by a variety of drugs, by hyperoxia, and by reflow following ischemia. However, much of what is known about the mechanisms by which reactive oxygen species kill cells has been developed through studies conducted *in vitro*, particularly isolated or cultured rat hepatocytes (1-5). Gerson *et al.* (3) reported recently that the toxicity of acetaminophen to cultured rat hepatocytes is potentiated by BCNU pretreatment, which was interpreted as evidence that acetaminophen kills hepatocytes through oxidative mechanisms, because the major known effect of BCNU treatment is the

Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; GSSG, glutathione disulfide; GSH, glutathione; GPT, glutamate pyruvate transaminase; GOT, glutamate oxalacetate transaminase; NPSH, nonprotein sulfhydryl; PSH, protein sulfhydryl.

0006-291X/87 $1.50

irreversible inhibition of glutathione reductase (6). In contrast, we found that BCNU pretreatment does not potentiate acetaminophen-induced hepatic necrosis *in vivo* and administration of acetaminophen produces no measurable oxidant stress, as evaluated by rates of biliary GSSG efflux, even in animals pretreated with BCNU (7). Obviously, conclusions from these data would be much clearer if BCNU could be shown to potentiate the hepatotoxicity of an agent that produces measurable oxidant stress *in vivo*.

Recently we have reported that diquat-mediated hepatic necrosis in male Fischer-344 rats appears to be produced through oxidant mechanisms, presumably initiated by the redox cycling metabolism of diquat with generation of excessive amounts of reactive oxygen species (8). Therefore, we now have examined the effects of BCNU pretreatment on the hepatotoxicity and biliary efflux of GSSG after diquat in Fischer-344 and Sprague-Dawley rats.

MATERIALS AND METHODS

Diquat was obtained as a generous gift from Dr. Ian Wyatt of Imperial Chemical Industries, Ltd. (Macclesfield, Cheshire, England). BCNU was provided similarly by Dr. Jim Keller of Bristol Laboratories (Syracuse, New York). Other reagents were purchased from Sigma Chemical Company (St. Louis, Missouri) or from Fisher Scientific (Houston, Texas). Animals were obtained from Harlan Sprague-Dawley Inc. (Houston, Texas) and kept in cages in a climate-controlled room with a 12-hour light-dark cycle and had access *ad libitum* to tap water and food. BCNU was administered i.p. in corn oil at a dose of 80 mg/kg at 4:00 pm and diquat administration i.p. in saline or biochemical determinations were begun between 08:00 and 09:00 the next morning. Control animals received equal volumes of vehicle. Biliary GSSG (9) and NPSH, PSH, GSSG reductase, plasma GPT and GOT were determined as we have described previously (8). Data are expressed as mean ± S.E. Plasma transaminase data was evaluated with the Mann-Whitney nonparametric rank sum test and other data evaluated with Student's t-test (10).

RESULTS AND DISCUSSION

Eighteen hours after 80 mg/kg of BCNU, hepatic glutathione reductase activities were depleted by 75 percent in both strains of rat (Table 1). The increase in hepatic NPSH in treated animals has been reported previously by Smith and Boyd (11), who employed an enzymatic assay and showed this increase to be predominantly GSH. BCNU-pretreated Fischer rats were significantly more sensitive to the hepatotoxic effects of diquat (Table 2) as expected, and the biliary excretion of GSSG induced by administration of 0.05 mmol/kg of diquat

TABLE 1. EFFECTS OF BCNU ON LIVERS OF FISCHER AND SPRAGUE-DAWLEY RATS

Strain	BCNU mg/kg	n	g liver / 100 g animal	NPSH nmol / mg tissue	PSH nmol / mg tissue	GSSG Reductase nmol NADPH / min mg tissue	GPT	GOT
Fischer	0	4	3.79 ± 0.05	6.01 ± 0.20	16.86 ± 0.52	6.04 ± 0.12	36 ± 2	137 ± 9
	80	4	3.11 ± 0.06[c]	9.90 ± 0.16[c]	17.34 ± 0.28	1.61 ± 0.05[c]	87 ± 16[a]	191 ± 21
Sprague-Dawley	0	4	4.64 ± 0.05	5.92 ± 0.33	17.70 ± 0.45	8.41 ± 0.25	33 ± 7	110 ± 6
	80	4	3.74 ± 0.24[b]	7.50 ± 0.72	18.62 ± 0.10	1.68 ± 0.21[c]	40 ± 4	81 ± 10

Fed male Fischer-344 or Sprague-Dawley rats (Harlan, Houston, Texas) were given BCNU 80 mg/kg i.p. in corn oil or an equal volume of corn oil alone (5 ml/kg). Eighteen hours later, animals were placed under light ether anesthesia and blood taken by retroorbital puncture for measurement of plasma transaminases. Animals were killed by decapitation, livers removed and weighed, and a portion of liver homogenized in 9 volumes of ice-cold 0.25 M sucrose. NPSH, PSH, and GSSG reductase were determined as described in Methods. Data given as mean ± SEM, n = 4. Plasma transaminases in Wroblewski-LaDue or Karmen units.

[a] Different from control by rank sum test, $p < 0.05$.

[b] Different from control by t-test, $p < 0.01$.

[c] Different from control by t-test, $p < 0.001$.

was greater in the BCNU-pretreated animals than in animals given only diquat (Figure 1). In addition, hepatic necrosis was observed in a few Sprague-Dawley rats (Table 2); four of the 42 Sprague-Dawley rats given both drugs showed markedly elevated plasma transaminase activities within six hours, and none of the four survived for 24 hours. Five additional animals showed modest increases in plasma transaminase activities (200-600 U/ml) at 24 hours. Hepatic necrosis in these animals was confirmed by histology (Smith and Mitchell, unpublished).

The potentiation of diquat hepatotoxicity in Fischer rats by BCNU pretreatment and inhibition of glutathione reductase is consistent with mediation of cell damage by reactive oxygen species. Burk *et al.* (12) have reported similar effects on diquat-initiated hepatic damage by inhibition of the glutathione peroxidase/reductase system through dietary selenium deficiency, which decreases hepatic glutathione peroxidase.

Although the data available suggest strongly that diquat produces hepatic necrosis through the generation of reactive oxygen species, the nature of the critical lesions is less clear. Oxidation of cellular thiols occurs, but depletion of NPSH is not extensive and measurable changes in hepatic PSH are

TABLE 2. EFFECT OF BCNU PRETREATMENT ON DIQUAT HEPATOTOXICITY

Strain	BCNU mg/kg	Diquat mmol/kg	Time hr	Survival	GPT U/ml	GOT U/ml
Fischer	0	0	6	4/4	56 ± 2	134 ± 14
	0	0.05	6	9/9	69 ± 10	175 ± 12
			24	9/9	186 ± 25	105 ± 7
	0	0.075	6	4/4	149 ± 55	260 ± 82
			24	4/4	320 ± 190	526 ± 345
	80	0	6	4/4	122 ± 56	258 ± 117
			24	4/4	216 ± 31	402 ± 46
	80	0.05	3	5/5	3750 ± 999[a]	6953 ± 1268[a]
			6	0/5	---	---
	80	0.075	2	7/7	3831 ± 1197[a]	6804 ± 1516[a]
			6	0/7	---	---
Sprague-Dawley	0	0	6	8/8	41 ± 9	94 ± 5
			24	8/8	42 ± 6	80 ± 11
	0	0.1	6	9/9	89 ± 13	211 ± 29
			24	5/9	52 ± 7	151 ± 48
	80	0	6	5/5	38 ± 6	179 ± 28
			24	4/5	56 ± 10	212 ± 39
	80	0.1	6	35/42	205 ± 129	545 ± 331
			24	31/42	48 ± 8	156 ± 18

Adult male Fischer-344 or Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Houston, Texas) were given 80 mg/kg of BCNU in corn oil or an equal volume of corn oil i.p. and 18 hours later were treated i.p. with diquat in saline or saline alone. Blood was collected by retroorbital puncture at the times indicated, centrifuged, and plasma transaminases measured. Early collection of blood from animals treated with BCNU and diquat was made necessary by animal mortality. Data are given as mean ± S.E.M. and evaluated statistically by Mann-Whitney rank sum test. [a]Plasma transaminase activities in animals receiving BCNU and diquat are higher ($p < 0.05$) than animals receiving the indicated dose of diquat alone.

not produced *in vivo* either in animals given 0.1 mmol/kg of diquat alone (6) or in animals given 0.05 mmol/kg of diquat after BCNU pretreatment (Table 3), in contrast to the mechanisms implicated by studies *in vitro* (1,2). However, the studies *in vivo* could not exclude the significance of protein S-thiolation in lobular or subcellular compartments.

From our initial studies it was not entirely clear that the failure to

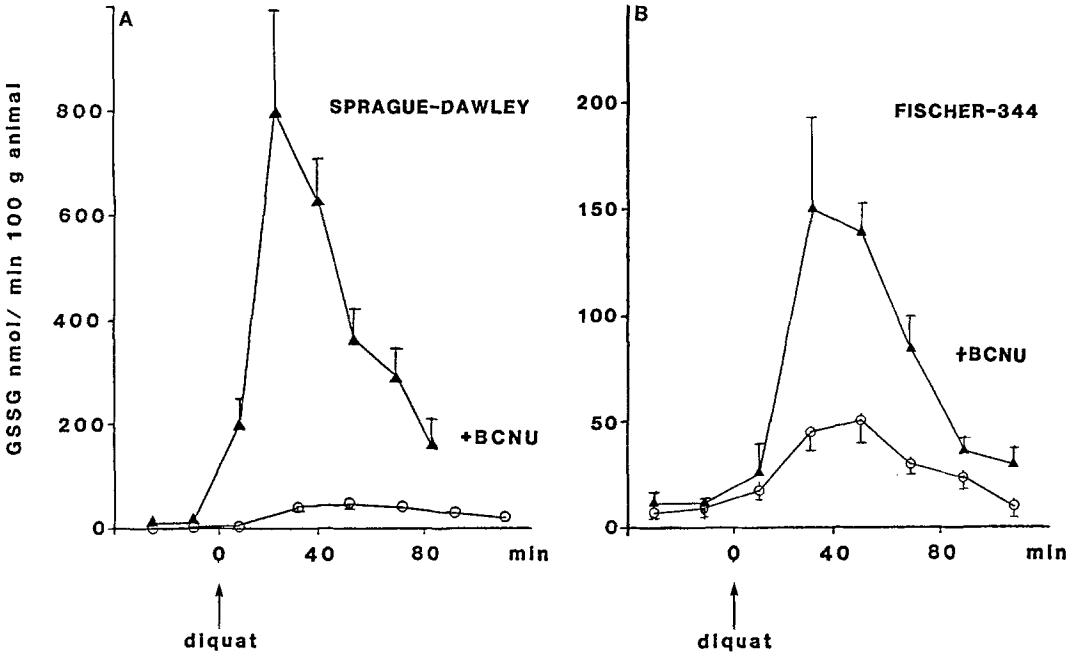

Figure 1. Effect of BCNU on biliary GSSG after diquat. Male Sprague-Dawley rats (A) or Fischer-344 rats (B) were given BCNU (80 mg/kg, i.p.) (▲) or an equal volume of corn oil alone (○) 18 hr before 50 mg/kg of pentobarbital for anesthesia and cannulation of their common bile ducts. Timed bile samples were collected before and after administration of diquat at 10 min. The Sprague-Dawley rats received 0.1 mmol/kg and the Fischer rats received 0.05 mmol/kg of diquat. Biliary GSSG was determined as we have described previously (6) and is expressed in GSH equivalents, mean ± S.E.M., n = 4. Note the difference in scale.

observe diquat-induced hepatic necrosis in the Sprague-Dawley rats was not the result of a lesser hepatic oxidant stress but comparable acute animal mortality, which prevented the useful investigation of responses at higher doses of diquat. The relative resistance of BCNU-pretreated Sprague-Dawley rats to diquat-initiated hepatic injury in spite of the dramatic increases in

TABLE 3. EFFECT OF BCNU PRETREATMENT ON HEPATIC NPSH AND PSH IN DIQUAT-TREATED FISCHER RATS

BCNU mg/kg	Diquat mmol/kg	n	NPSH nmol/mg tissue	PSH nmol/mg tissue
0	0.05	4	4.52 ± 0.52	16.70 ± 0.73
80	0.05	4	4.32 ± 0.56	16.57 ± 0.98

Hepatic NPSH and protein sulfhydryl (PSH) contents were determined in the animals from which the data in Figure 1 was obtained two hours after diquat was administered.

biliary GSSG efflux (Figure 1) do not support hypotheses of oxidant stress-induced cell death through cellular thiol oxidation. Lobular or subcellular compartmentation of thiol oxidation is not excluded by these studies, but shifts in cellular thiol/disulfide equilibria would appear unlikely to be responsible for initiation of cell death.

In addition to the quantitatively massive oxidation of cellular thiols produced by hepatotoxic doses of diquat, peroxidation of lipids as measured by hepatic content of 11,12, and 15-hydroxyeicosatetraenoates (HETEs) occurs (8), indicating that peroxidation of cellular lipids may be critical in the initiation of cell death. This speculation is supported further by our observations that hepatic microsomes isolated from Fischer rats given hepatotoxic doses of diquat show a markedly increased permeability to calcium (13). Loss of homeostatic control of intracellular calcium concentrations has been proposed as a critical step in the progression from cell injury to the initiation of cell death (14) and changes in permeability to calcium through peroxidation of membrane lipids may represent a significant mechanism of cell death initiated by reactive oxygen species or by free radicals (15). Further support for the hypothesis that lipid peroxidation is causal in diquat-induced hepatic necrosis are our recent observations that hepatotoxic doses of diquat increase ethane and pentane expiration in Fischer rats, an effect that is inhibited in parallel with protection against hepatic damage by treatment with the iron chelator desferrioxamine; conversely hydrocarbon expiration and hepatic injury in response to diquat are potentiated by pretreatment with $FeSO_4$ (16).

Thus, the evidence available at present suggest that lipid peroxidation, and not protein thiol oxidation, may represent the critical molecular lesion in diquat-initiated, reactive oxygen-mediated hepatic necrosis.

ACKNOWLEDGMENT

The work was supported by National Institutes of Health Grant GM 34120. The support and advice of Dr. Jerry R. Mitchell are gratefully acknowledged.

REFERENCES

1. DiMonte, D., Bellomo, G., Thor, H., Nicotera, P. and Orrenius, S. (1984) Arch. Biochem. Biophys. 235, 343-350.
2. DiMonte, D., Ross, G., Bellomo, G., Eklow, L. and Orrenius, S. (1984) Arch. Biochem. Biophys. 235, 334-342.
3. Gerson, R.J., Casini, A., Gilfor, D., Serroni, A. and Farber, J.L. (1985) Biophys. Res. Comm. 126, 1129-1137.
4. Starke, P.E. and Farber, J.L. (1985) J. Biol. Chem. 260, 86-92.
5. Starke, P.E. and Farber, J.L. (1985) J. Biol. Chem. 260, 10099-10104.
6. Frischer, H. and Ahmad, T. (1977) J. Lab. Clin. Med. 89, 1080-1091.
7. Smith, C.V. and Mitchell, J.R. (1985) Biochem. Biophys. Res. Comm. 133, 329-336.
8. Smith, C.V., Hughes, H., Lauterburg, B.H. and Mitchell, J.R. (1985) J. Pharmacol. Exp. Med. 235, 172-177.
9. Lauterburg, B.H., Smith, C.V., Hughes, H. and Mitchell, J.R. (1984) J. Clin. Invest. 73, 124-133.
10. Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, pp. 3-134, Iowa State University Press, Ames.
11. Smith, A.C. and Boyd, M.R. (1984) J. Pharmacol. Exp. Ther. 229, 658-663.
12. Burk, R.J., Lawrence, R.A. and Lane, J.M. (1980) J. Clin. Invest. 65, 1024-1031.
13. Tsokos-Kuhn, J.O., Smith, C.V., Hughes, H., McMillin-Wood, J.B. and Mitchell, J.R. (1985) Pharmacologist 27, 157.
14. Bellomo, G. and Orrenius, S. (1985) Hepatology 51, 876-882.
15. Tsokos-Kuhn, J.O., Smith, C.V., Mitchell, J.R., Tate, C.A. and Entman, M.L. (1986) Mol. Pharmacol. 30, 444-451.
16. Smith, C.V., Hughes, H., Kuhn, J.O. and Mitchell, J.R. (1986) Pharmacologist 28, 207.