Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 329-336

ACETAMINOPHEN HEPATOTOXICITY IN VIVO IS NOT ACCOMPANIED BY OXIDANT STRESS

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Received October 4, 1985

SUMMARY. Hepatotoxic doses of acetaminophen in Fischer 344 rats did not increase biliary efflux of oxidized glutathione. Pretreatment of the animals with bis(2-chloroethyl)-N-nitrosourea inhibited hepatic glutathione reductase by 73 percent but did not potentiate the hepatotoxicity of acetaminophen and did not produce an increase in biliary efflux of oxidized glutathione in response to acetaminophen. Hepatic protein thiol content was not depleted by acetaminophen. A proposed role for oxidant stress mechanisms mediated either by reactive oxygen species or by the direct oxidant action of a reactive metabolite in acetaminophen-induced hepatotoxicity is unsubstantiated and unlikely.

INTRODUCTION. The association between the hepatotoxicity of acetaminophen and the extent to which acetaminophen metabolites become covalently bound to tissue protein was reported initially in 1973 (1,2). In the numerous studies of acetaminophen hepatotoxicity that have been reported subsequently, several have been claimed to demonstrate a "dissociation" of covalent binding from cell death but these have been conducted primarily in vitro (3,4). To our knowledge, there have been no reports of acetaminophen-induced hepatic necrosis in vivo in the absence of measurable covalent binding; rather, the lack of a linear correlation between the extent of binding and the extent of cell death has been shown.

A major problem in the interpretation of the results of studies in vivo has been the failure to correct covalent binding data for the distinctive hepatic swelling produced by entrapment of plasma and subsequently of red blood cells (5-7). In addition, the magnitude of the tissue lesion in vivo reflects not only the cellular toxicity initiated by the reactive metabolite, but also

Abbreviations: GSSG, glutathione disulfide; NPSH, nonprotein sulfhydryls; PSH, protein sulfhydryls; BCNU, 1,3-bis(2-chloroethyl)-N-nitrosourea; APAP, acetaminophen.

0006-291X/85 $1.50

physiologic responses such as zonal ischemia resulting from the initial lesion plus the swelling. Recent data also implicate changes in calcium homeostasis as contributing to acetaminophen toxicity since inhibition of ATP-dependent calcium accumulation by plasma membrane vesicles (8), as well as inhibition of calcium-dependent ATPase (9), occur after acetaminophen administration *in vivo*.

Nevertheless, a causal role for covalent binding to hepatic protein in acetaminophen-induced hepatotoxicity has not been proven, and other hypotheses merit evaluation. Recently, Gerson *et al.* (4) reported that BCNU-pretreatment potentiated the toxicity of acetaminophen to cultured hepatocytes and concluded that acetaminophen was killing cultured hepatocytes by oxidant stress mechanisms. However, no chemical or biochemical documentation of an oxidant stress or of its potentiation by BCNU pretreatment was provided.

We have examined the effects of BCNU pretreatment on acetaminophen hepatotoxicity *in vivo* to investigate the possibility that an otherwise hidden oxidant stress response might be magnified to the point that it would be detectable. The present data do not support the existence of an oxidant stress associated with acetaminophen metabolism and hepatotoxicity *in vivo*.

MATERIALS AND METHODS. Adult male Fischer 344 rats were purchased from Harlan Industries (Houston, Texas) and allowed free access to tap water and food (Wayne Rodent Chow). BCNU (a generous gift from Bristol-Meyers) was administered i.p. 80 mg/kg in corn oil 18 h prior to acetaminophen, which was given p.o. in saline solution between 8:00 and 9:00 a.m. Control animals received equal volumes of vehicle. Animals were anesthetized with ether and blood obtained by retroorbital puncture was centrifuged to provide plasma for determination of transaminase activities with commercial kits (Sigma). Hepatic nonprotein sulfhydryl (NPSH) and protein sulfhydryl (PSH) contents (10) and biliary efflux of GSSG (11) were measured as we have described previously. Data are presented as mean $\pm$ SEM. Statistical significance of differences in treatment groups was evaluated by Mann-Whitney rank sum test or the unpaired Student's t-test (12).

RESULTS AND DISCUSSION. For studies of possible hepatic oxidant stress *in vivo*, excretion of GSSG into bile and plasma have been particularly useful (11,13). Biliary excretion of GSSG was stimulated markedly by redox compounds like diquat and GSSG excretion is such an extremely sensitive index of intracellular oxidant stress that 10-20 fold increases are produced before the protective

mechanisms of the cell are overcome sufficiently to produce necrosis. Both the hepatic necrosis and the biliary efflux of GSSG in response to diquat were potentiated dramatically by pretreatment of the animals with BCNU (14). In contrast, administration of hepatotoxic doses of acetaminophen to male Fischer-344 rats did not increase biliary GSSG at any dose, even in animals pretreated with BCNU (Fig. 1). Further, BCNU pretreatment did not potentiate the hepatotoxicity of acetaminophen (Table 1). The decrease in biliary GSSG observed following administration of acetaminophen reflects depletion of hepatic GSH, and biliary GSH content decreases with a similar time course (data not shown). However, it should be noted that biliary GSSG has decreased at a time when substantial hepatic GSH is present and should have been able to reveal an oxidant stress, either by formation of GSSG directly or by thiol-disulfide exchange with oxidized PSH.

Di Monte et al. proposed recently that menadione kills isolated hepatocytes by oxidation of PSH once virtually all NPSH has been depleted

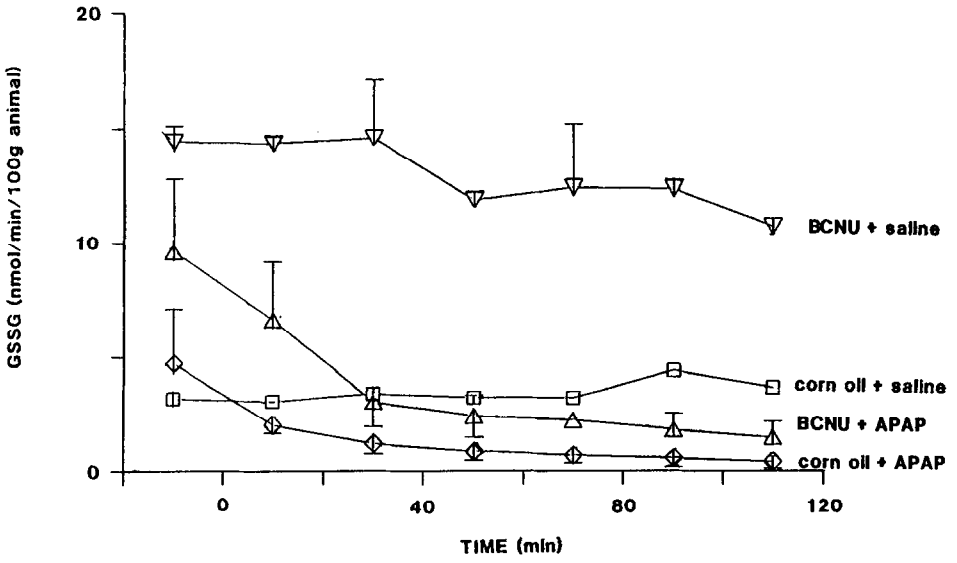

Figure 1. Effect of acetaminophen on biliary efflux of GSSG. Male Fischer-344 rats were treated with BCNU (80 mg/kg, i.p.) in corn oil or an equal volume of corn oil alone 18 h prior to pentobarbital (50 mg/kg) anasthesia and bile duct cannulation. Acetaminophen (1.5 g/kg) in saline or an equal volume of saline was administered at time 0. Bile was collected in 20 min fractions and GSSG measured as described in (11). Data are expressed as mean + SEM.

TABLE 1. EFFECT OF BCNU PRETREATMENT ON ACETAMINOPHEN HEPATOTOXICITY

BCNU mg/kg	APAP mg/kg	n	GPT U/ml	GOT U/ml
0	1000	9	211 ± 123	459 ± 274
80	1000	9	159 ± 20	386 ± 45
0	1500	4	1664 ± 494	3431 ± 1234
80	1500	4	174 ± 36 *	146 ± 55 *
0	2000	4	2981 ± 871	4898 ± 869
80	2000	4	1386 ± 890	3934 ± 2762

Male Fischer 344 rats were given BCNU i.p. in corn oil or an equal volume of corn oil 18h prior to acetaminophen p.o. Blood was collected by retroorbital puncture 24h after acetaminophen and plasma transaminases determined with kits purchased from Sigma. Animal mortality was zero. Data are given as mean ± SEM. *Different from animals not receiving BCNU by Mann-Whitney rank sum test, $p<0.05$.

(15,16). A similar mechanism for acetaminophen hepatotoxicity could be proposed, but would require an explanation for the lack of GSH oxidation by acetaminophen, in contrast to the substantial GSH oxidation observed for menadione. Additional evidence against a role for PSH oxidation in acetaminophen hepatoticity is that hepatic PSH is not depleted (Table 2). Although hepatic PSH is lower, per mg tissue, in acetaminophen-treated animals at 3 h, this apparent difference disappears when hepatic PSH is expressed per 100 g animal body weight. Walker et al. (6) have reported that in mice treated with acetaminophen, the hepatic swelling at 3 h is mostly plasma water, whereas by 6 h a marked increase in hepatic hemoglobin content is noted, indicating entrapment of red blood cells. A similar sequence of events would provide an explanation for the apparent changes in hepatic PSH (Table 2). Swelling at 3 h dilutes hepatic PSH with plasma water but does not alter total hepatic PSH content (PSH content of venous plasma measured by our methods gave 0.16 ± 0.01 nmol/µl plasma, n=4). The significant entrapment of red blood cells by 6 h could account for the observed increase in total hepatic PSH. We interpret the data as evidence that hepatocellular PSH content is not altered by

TABLE 2. EFFECT OF ACETAMINOPHEN ON HEPATIC PROTEIN SULFHYDRYL CONTENT

APAP	TIME		NPSH		PSH		GPT
			nmole	µmole	nmole	µmole	U
mg/kg	h	n	mg tissue	100g animal	mg tissue	100g animal	ml
0	3	4	3.60 ± 0.22	10.42 ± 0.67	16.80 ± 0.47	48.7 ± 1.7	36 ± 4
1500	3	6	0.27 ± 0.08 ***	1.01 ± 0.30 ***	14.11 ± 0.47 **	52.4 ± 4.0	76 ± 25
0	6	4	3.23 ± 0.38	9.16 ± 1.10	16.77 ± 0.98	47.5 ± 2.8	30 ± 2
1500	6	6	0.38 ± 0.41 ***	4.80 ± 1.62	14.17 ± 0.70	57.9 ± 2.4 **	625 ± 87 *

Adult male Fischer 344 rats were fasted 24h prior to p.o. administration of APAP or an equal volume of saline. Blood was drawn by retroorbital puncture, animals killed by decapitation and hepatic NPSH and PSH content determined as described in Methods. Data are mean ± SEM. Different from control by unpaired t-test: ** $p<0.025$; *** $p<0.001$. Different from control by Mann-Whitney rank sum test: * $p<0.05$. Note: measurement of venous plasma PSH content by this method gave 0.16 ± 0.01 nmol/µl (n=4).

acetaminophen. In any event, the 16 percent decrease in hepatic PSH per mg liver is less than the 40 per cent loss identified by the studies of Di Monte et al. (15,16) required to kill isolated hepatocytes.

The mass of data available at this time thus do not support the existence of an oxidant stress produced in vivo by acetaminophen, and certainly not one of sufficient magnitude to produce hepatic necrosis. The nature of the potentiation by BCNU of acetaminophen killing of cultured hepotocytes reported by Gerson et al. (4) is not clear, but documentation of an acetaminophen-induced oxidant stress in that system and of its potentiation by BCNU should be provided for the hypothesis to be considered relevant even for cultured hepatocytes.

Although oxidant mechanisms apparently do not contribute to acetaminophen hepatoxicity, an oxidant stress response still would be expected if N-acetyl-p-benzoquinoneimine (NAPQI) (II) is quantitatively a free intermediate in the metabolism of acetaminophen (Fig. 2). The reaction of synthetic NAPQI

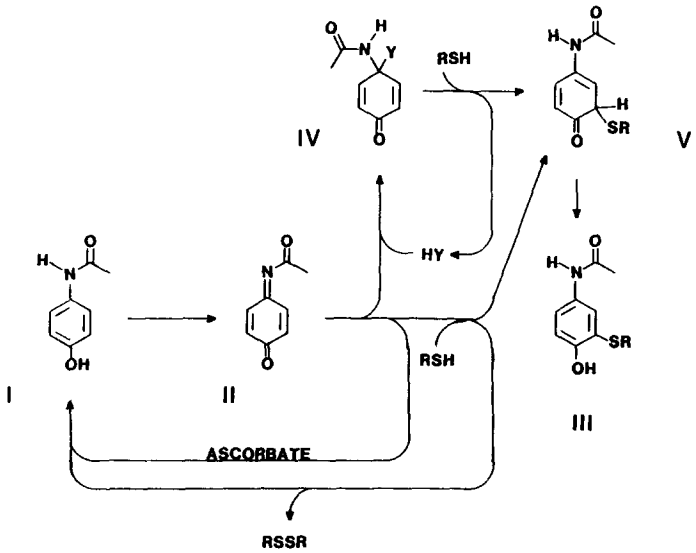

Figure 2. A scheme of acetaminophen metabolism attempting to account for the absence of observable GSSG formation or of inhibition of acetaminophen covalent binding by ascorbate or by the D-isomer of N-acetylcysteine, by invoking the preferential formation of an intermediate IV capable of alkylation of RSH, but not active as an oxidant. Glutathione transferases are candidates for the HY function. Although ipso addition of HY to the imine function is depicted, 1,4- addition could serve the same function.

with GSH *in vitro* produces both the GSH adduct (III) and acetaminophen (I) by alkylation and reduction, respectively. The lack of measurable GSSG formation after acetaminophen *in vivo* and, as recognized by Miller and Jollow (17), the inability of ascorbate to prevent covalent binding of acetaminophen *in vivo* or in isolated hepatocytes (17), in contrast to the inhibition by ascorbate of binding noted in microsomal and in chemical systems (18,19), suggest that the chemically reactive metabolite of acetaminophen may not be formally the quinoneimine, but may be a species (IV) derived from addition of NAPQI to a nucleophilic center on a GSH-S-transferase, which in turn could serve as a leaving group for alkylation of GSH or PSH by the acetaminophen moiety. This hypothesis also would explain the failure of strong reducing compounds, such as N-acetyl-D-cysteine (20), in contrast to the L-isomer (1), to protect against acetaminophen hepatic necrosis, because the D-isomer is not a substrate for gamma-glutamylcysteine synthetase and hence will not support glutathione synthesis as will the L-isomer (21).

ACKNOWLEDGEMENT. The generous support of National Institutes of Health grant GM12148 is gratefully acknowledged.

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