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1,3-(2-Chloroethyl)-1-nitrosourea Potentiates the Toxicity of Acetaminophen Both in the Phenobarbital-Induced Rat and in Hepatocytes Cultured from Such Animals

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SUMMARY

The toxicity of acetaminophen was studied in hepatocytes cultured from phenobarbital-induced male rats. Such cells were less sensitive to acetaminophen than similar ones cultured from animals induced with 3-methylcholanthrene. In both cases, the toxicity of acetaminophen depended on its metabolism. Inhibition of glutathione reductase with 1,3-(2-chloroethyl)-1-nitrosourea (BCNU) potentiated the toxicity of acetaminophen in the presence or absence of 100 mM acetone, an agent that activates the mixed function oxidation of the toxin. BCNU enhanced the rate and extent of the depletion of GSH in the presence or absence of acetone. Pretreatment of the hepatocytes with the ferric iron chelator deferoxamine or addition to the culture medium of the antioxidant *N,N'*-diphenyl-*p*-phenylenediamine prevented the toxicity of acetaminophen in the presence of BCNU whether or not there was acetone in the cultures. BCNU similarly potentiated the hepatotoxicity of acetaminophen in the intact, phenobarbital-induced rat. These data indicate that the mechanism of the killing of hepatocytes induced with phenobarbital is similar to that reported previously with hepatocytes prepared from animals induced with 3-methylcholanthrene. In both cases it would seem that the liver cells are killed by acetaminophen as a result of an oxidative stress that accompanies the metabolism of this hepatotoxin.

Isolated hepatocytes have become an important tool in the analysis of the mechanisms of action of toxic chemicals. Suspended or cultured hepatocytes can clearly be manipulated to a greater extent than can the same cells in the intact animal. This expediency is in large part responsible for the many recent advances in our understanding of the mechanisms of toxic cell injury. On the other hand, however, the ease with which hepatocytes can be manipulated is responsible for a variety of experimental conditions that have not always yielded the same conclusions.

A case in point is the recent studies of the mechanisms whereby acetaminophen causes liver injury. Acetaminophen is a widely used analgesic that produces liver necrosis in both rodents and humans. We have been using cultures of rat hepatocytes as a model system with which to explore the mechanism of the hepatotoxicity of acetaminophen. In our cell culture system, the sensitivity of hepatocytes to acetaminophen is induced by pretreatment of the animals with 3-methylcholanthrene (1–3). Inhibition of glutathione reductase by BCNU, known to sensitize hepatocytes to an oxidative stress (4, 5), potentiates the toxicity of acetaminophen without increasing either the metabolism of acetaminophen or the covalent binding of acetaminophen metabolites (1, 3). Superoxide dismutase, catalase, and mannitol prevent the cell killing (2). Pretreatment of the hepatocytes with the ferric iron chelator deferoxamine, known to reduce the sensitivity of hepatocytes to an oxidative stress (6, 7), prevents the cell killing without reducing covalent binding (1, 2). Such data document the participation of oxygen radicals in the killing of cultured hepatocytes by acetaminophen in this model and suggest that hydroxyl radicals generated by an iron-catalyzed Haber-Weiss reaction mediate the cell injury.

By contrast, a recent investigation of the mechanism of action of acetaminophen that utilized suspensions of hepatocytes isolated from phenobarbital-induced male rats did not observe potentiation of the cell killing by BCNU (8). In addition, deferoxamine had no effect on the toxicity of acetaminophen. It was concluded that, under the conditions used, acetaminophen causes cytotoxicity without involvement of an oxidative stress (8).

Differences in the experimental conditions may relate to the differing conclusions (1, 2, 8). In addition to the use of suspen-

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ABBREVIATIONS: BCNU, 1,3(2-chloroethyl)-1-nitrosourea; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; LDH, lactic dehydrogenase; DEM, diethylmaleate.

sions of phenobarbital-induced hepatocytes under an atmosphere of 95% O_2/5% CO_2, Porubek *et al.* (8) included 100 mM acetone in all of the incubations. The present report was prompted by an interest in determining whether the differing conclusions (1, 2, 8) are related to these differences in the experimental conditions. The data reported here clearly indicate that the killing by acetaminophen of hepatocytes cultured from phenobarbital-induced male rats is potentiated by BCNU in the presence and absence of 100 mM acetone. In addition, it is shown that BCNU similarly potentiates the hepatotoxicity of acetaminophen in the intact rat. The reason for the previous (8) inability to observe such an effect of BCNU is discussed.

Materials and Methods

Male Sprague-Dawley rats (175–225 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats pretreated with sodium phenobarbital were given 80 mg/kg/day by intraperitoneal injection for 3 days. Rats pretreated with 3-methylcholanthrene (Sigma Chemical Co., St. Louis, MO) were given a single intraperitoneal injection of 25 mg/kg as a 10 mg/ml solution, in corn oil, on the day before preparation of hepatocytes. All animals were fasted overnight before use.

Isolated hepatocytes were prepared by collagenase perfusion according to the method of Seglen (9). Yields of 2–4 × 10^8 cells/liver with 85–90% viability (trypan blue exclusion) were routinely obtained. The hepatocytes were plated in plastic 25-cm flasks (Corning, Corning, NY) at a density of 1.33 × 10^6 cells/flask in Williams E medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Hazelton Research Products Co., Lenexa, KS), 10 units/ml penicillin, 10 μg/ml streptomycin, and 0.02 units/ml insulin. After incubation at 37° in 5% CO_2/95% air for 2 hr, the cultures were rinsed twice to remove unattached dead cells, fresh medium was added, and the cells were incubated for another 1.5 hr. In some cases 20 mM deferoxamine (Ciba Pharmaceutical, Summit, NJ) was added to the culture medium during the last hour of this second period of incubation. In all cases, fresh medium containing the additions indicated in the text was added once more (the cells were in culture at this point for 3.5 hr). Viability of the cultured cells was assayed by the release of LDH (10).

Acetaminophen (Sigma) was dissolved in dimethyl sulfoxide. SKF 525-A (SmithKline Beckman, Philadelphia, PA) and β-naphthoflavone (Sigma) were dissolved in dimethyl sulfoxide and added to the cultures at a final concentration of 10 μM. BCNU (Bristol Laboratories, Syracuse, NY) was dissolved in dehydrated ethanol and added directly to the cultures to a final concentration of 50 μM. DPPD (Eastman, Rochester, NY) was dissolved in dimethyl sulfoxide and added to the cultures at a final concentration of 2 μM. GSH was measured by adaptation of the method of Sedlak and Lindsay (11).

The covalent binding of [^{3}H]acetaminophen metabolites to total hepatocyte proteins was measured after incubating the cells for 20 hr with 1 μCi/ml of [^{3}H]acetaminophen, 9.3 Ci/mmol (New England Nuclear, Boston, MA). The final concentration of acetaminophen in the cultures is shown in the legend to Fig. 2. At the end of the incubation, the medium was removed and 5 ml of 10% trichloracetic acid were added. The cells were scraped from the flasks, the suspension was recovered by centrifugation, and the proteins were prepared according to the method of Rao and Recknagel (12). The dry protein residue was weighed in tared scintillation vials, dissolved in 1 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL) and counted in 15 ml of a toluene-based solution.

Results

Sensitivity of phenobarbital-induced hepatocytes to acetaminophen. Cultured hepatocytes prepared from male rats and exposed to as much as 10 mM acetaminophen showed no loss of viability over an 18-hr time course. Sensitivity to acetaminophen was induced by pretreatment of the animals with either phenobarbital or 3-methylcholanthrene. However, these compounds differed in their ability to sensitize hepatocytes to acetaminophen. As shown in Fig. 1, the hepatocytes from an animal given a single dose of 25 mg/kg 3-methylcholanthrene died at lower concentrations of acetaminophen than did those prepared from an animal treated with 80 mg/kg of phenobarbital for 3 days. This greater sensitivity to acetaminophen of hepatocytes prepared from animals induced with 3-methylcholanthrene as opposed to phenobarbital agrees with the similar effect of these same compounds in intact rodents (13, 14).

This differing sensitivity to acetaminophen of hepatocytes cultured from animals induced with either 3-methylcholanthrene or phenobarbital was reflected in the covalent binding of metabolites of [^{3}H]acetaminophen to total proteins. In the experiment illustrated in Fig. 2, 3-methylcholanthrene- or phenobarbital-induced hepatocytes were exposed to increasing concentrations of [^{3}H]acetaminophen. After 20 hr the extent of the covalent binding of acetaminophen metabolites to total cell proteins was determined. The extent of binding was greater in the hepatocytes from 3-methycholanthrene-induced animals, an observation in agreement with the greater sensitivity of these same cells to acetaminophen, illustrated in Fig. 1.

The killing by acetaminophen of hepatocytes prepared from animals induced with either 3-methylcholanthrene or phenobarbital depended on the metabolism of the toxin. SKF 525-A inhibits the mixed function oxidase activity that depends on

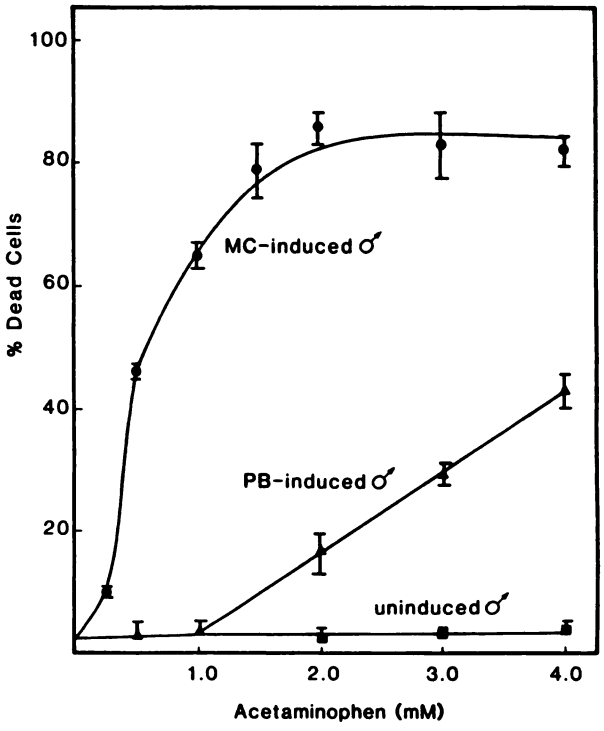

Fig. 1. Dose dependence of the killing of cultured hepatocytes by acetaminophen. Cultured hepatocytes prepared from either uninduced male rats (■), phenobarbital-induced male rats (▲), or 3-methylcholanthrene-induced male rats (●) were treated with increasing concentrations of acetaminophen. The viability of the cells was determined after 18 hr by measuring the content of LDH in the culture medium. The results are the mean ± standard deviation of the determinations on three separate cultures.

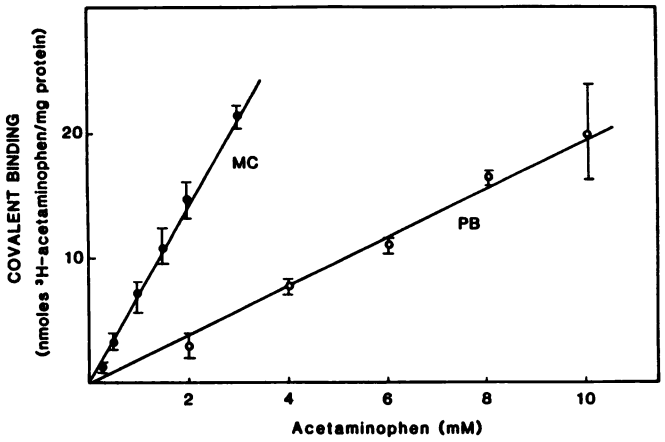

Fig. 2. Dose dependence of the covalent binding of [^{3}H]acetaminophen metabolites to total cellular proteins. Hepatocytes from phenobarbital- (○) or 3-methylcholanthrene-induced (●) male rats were incubated with [^{3}H]acetaminophen at the concentrations indicated. After 20 hr the extent of the covalent binding of acetaminophen metabolites to total cellular protein was measured. The results are the mean ± standard deviation of the determinations on three separate cultures.

TABLE 1

Dependence on metabolism of the killing of cultured hepatocytes by acetaminophen

Isolated hepatocytes were prepared from male rats pretreated with either a single injection of 25 mg/kg of 3-methylcholanthrene or three daily injections of 80 mg/kg of phenobarbital. The cells were cultured for 3.5 hr before treatment with acetaminophen. The 3-methylcholanthrene cells were treated with 2 mM and phenobarbital cells with 7.5 mM acetaminophen. The extent of cell killing was assessed after 24 hr. Viability of the cells was determined by the release of LDH into the culture medium. The results are the mean ± standard deviation of the determinations on three separate cultures.

Treatment	Dead Cells	
	3-Methylcholanthrene	Phenobarbital
	% of total	
1. APAP[a]	74 ± 4	54 ± 5
2. APAP plus SKF 525-A	55 ± 4	9 ± 2
3. APAP plus β-naphthoflavone	15 ± 2	57 ± 8

[a] APAP, acetaminophen.

the cytochrome P_{450} induced by phenobarbital. By contrast, β-naphthoflavone inhibits the mixed function oxidase activity that depends on the cytochrome P_{450} induced by 3-methylcholanthrene. As shown in Table 1, 10 μM SKF 525-A reduced by 90% the number of phenobarbital-induced hepatocytes killed by 7.5 mM acetaminophen, whereas it had only a slight effect on the killing of 3-methylcholanthrene-induced cells by 2.0 mM acetaminophen. By contrast, 10 μM β-naphthoflavone effectively protected 3-methylcholanthrene-induced hepatocytes from the toxicity of acetaminophen but was without effect on phenobarbital-induced cells.

Potentiation by BCNU and acetone of the toxicity of acetaminophen. BCNU potentiates the killing by acetaminophen of hepatocytes cultured from animals that were induced with 3-methylcholanthrene (1–3). We show here that the toxicity of acetaminophen for hepatocytes prepared from animals that were induced with phenobarbital is similarly potentiated by BCNU. In addition, this potentiation by BCNU occurs in the presence or absence of acetone. Isolated hepatocytes were placed in culture for 2 hr washed, and given fresh medium. After another 90 min, the cells were washed again, divided into four groups and treated as shown in Fig. 3. At the end of 4 hr the viability of the cells was measured by the amount of LDH released into the medium.

Up to 7.5 mM acetaminophen alone (Fig. 3) had no effect on viability. By contrast, in the presence of 100 mM acetone, acetaminophen killed a little more than one third of the hepatocytes within 4 hr (Fig. 3). With 100 mM acetone, the cell killing was maximal with 2.5 mM acetaminophen. No further increase in the number of dead cells was seen with 5 and 7.5 mM acetaminophen. This result agrees closely with a previous report using suspensions of phenobarbital-induced hepatocytes, in which the formation of the glutathione conjugate, a metabolite that reflects oxidative metabolism, was maximal with 2 mM acetaminophen (14).

Like acetone, BCNU also potentiated the toxicity of acetaminophen for hepatocytes prepared from phenobarbital-induced animals (Fig. 3). Whereas 5 mM acetaminophen in the presence of 50 μM BCNU killed 50% of the hepatocytes, as noted above there was no cell killing in the absence of BCNU. When the hepatocytes were treated with both BCNU and acetone, the toxicity of acetaminophen was further enhanced (Fig. 3).

In the presence of both BCNU and acetone in the culture medium, 2.5 mM acetaminophen killed over 70% of the hepatocytes. With either BCNU or acetone alone, only 40% of the cells were killed by 2.5 mM acetaminophen. Thus, the presence of both 100 mM acetone and 50 μM BCNU has an additive effect on the toxicity of acetaminophen. This result is consistent with the fact that acetone activates the metabolism of acetaminophen (15), whereas BCNU acts by sensitizing the hepatocytes to the hydrogen peroxide that presumably results from this metabolism.

Effect of BCNU on GSH metabolism. The effect of

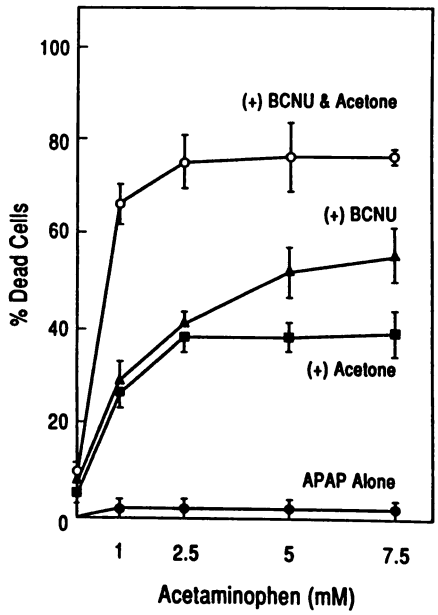

Fig. 3. Potentiation by BCNU of the toxicity of acetaminophen in the presence and absence of 100 mM acetone. Hepatocytes prepared from a male rat that was pretreated with phenobarbital were treated with increasing concentrations of acetaminophen (*APAP*) alone (●), acetaminophen plus 100 mM acetone (■), acetaminophen plus 50 μM BCNU (▲), or acetaminophen plus acetone and BCNU (○). The viability of the cells was measured 4 hours later. Acetone alone at 100 mM had no effect on the viability of the cells. The results are the mean ± standard deviation of the determinations on three separate cultures.

BCNU on the toxicity of acetaminophen is reflected in changes in the metabolism of GSH. Fig. 4 illustrates the time course of the changes in GSH in hepatocytes treated with 5 mM acetaminophen in the presence or absence of BCNU. In the absence of BCNU, there was again no cell killing with 5 mM acetaminophen over the 4 hr of the study. With BCNU, 43% of the cells were killed by 4 hr.

The control cells (no acetaminophen or BCNU) exhibited a steady increase in GSH content over the 4 hr of the experiment. This rise in GSH is similar to the response of hepatocytes prepared from animals that were induced by 3-methylcholanthrene (3). The presence of 5 mM acetaminophen in the culture medium reduced both the rate and the extent of the increase in the GSH content. When the hepatocytes were treated with both 5 mM acetaminophen and 50 μM BCNU (Fig. 4), the GSH content of the hepatocytes declined and was lower at every time, compared with cells treated with acetaminophen alone.

Treatment of the hepatocytes with BCNU alone reduced the GSH content of the cells, and the effects of BCNU and acetaminophen on GSH metabolism were essentially additive. Table 2 details the GSH content of the hepatocytes 1, 2, and 4 hr after treatment with BCNU alone (*line 2*), 5 mM acetaminophen alone (*line 3*), or BCNU and acetaminophen (*line 4*). The deficit in GSH content of the cells treated with BCNU and acetaminophen is essentially the sum of the effects of each agent when present alone.

However, the potentiation of the cell killing by BCNU can not be attributed to the lowered GSH content of the hepatocytes. Table 2 indicates the effect of treating the cells with 40 μM DEM alone (*line 5*) or together with 5 mM acetaminophen (*line 6*). With 40 μM DEM, the GSH content of the hepatocytes after 1 hr was actually lower than with 50 μM BCNU alone (*line 2* versus *line 5*). Furthermore, in the presence of DEM and acetaminophen, the GSH content of the cells after 4 hr

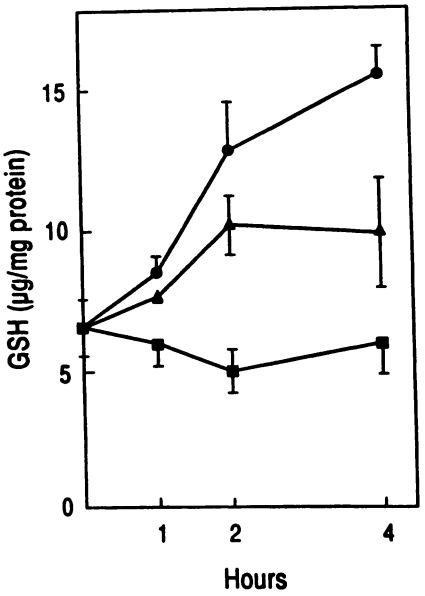

Fig. 4. Effect of BCNU on the metabolism of GSH in hepatocytes intoxicated with acetaminophen. Hepatocytes cultured from a male rat pretreated with phenobarbital were left untreated (●) or given 5 mM acetaminophen (▲) or acetaminophen plus 50 μM BCNU (■). At the times indicated, the GSH content of the hepatocytes was measured. The results are the mean ± standard error of the determinations on three separate cultures.

TABLE 2

GSH content of hepatocytes treated with BCNU and acetaminophen

Hepatocytes were isolated from male rats pretreated with three daily injections of 80 mg/kg of phenobarbital. After the cells were in culture for 3.5 hr they were treated as detailed below. At the times indicated, the GSH content of the hepatocytes was measured. The results are the mean ± standard deviation of the determinations on three separate cultures.

Treatment	GSH content			
	0 hr	1 hr	2 hr	4 hr
	μg/mg of protein			
1. Control	6.5 ± 1	8.5 ± 1	12.7 ± 2	15.4 ± 1
2. BCNU (50 μM)		5.9 ± 1	8.2 ± 1	10.6 ± 1
3. APAP[a] (5 mM)		7.6 ± 1	10.0 ± 1	9.7 ± 2
4. APAP and BCNU		5.9 ± 1	4.9 ± 1	5.7 ± 1
5. DEM (40 μM)		4.0 ± 1		13.5 ± 2
6. APAP and DEM				5.9 ± 1

[a] APAP, acetaminophen.

TABLE 3

Effect of diethylmaleate on the toxicity of acetaminophen

Hepatocytes were isolated from male rats pretreated with three daily injections of 80 mg/kg of phenobarbital. After the cells were in culture for 3.5 hr they were treated as detailed below. After 4 hr the viability of the cells was measured by the release of LDH into the culture medium. The results are the mean ± standard deviation of the determinations on three separate cultures.

Treatment	Dead Cells
	% of total
1. Control	2 ± 1
2. APAP[a] (5 mM)	1 ± 1
3. BCNU (50 μM)	4 ± 1
4. APAP plus BCNU	43 ± 1
5. DEM (40 μM)	2 ± 1
6. APAP plus DEM	4 ± 1

[a] APAP, acetaminophen.

(*line 6*) was identical to that with BCNU and acetaminophen at this time (*line 4*). However, DEM did not potentiate the toxicity of acetaminophen. Table 3 shows that the presence of DEM and acetaminophen had no effect on the cell viability, whereas BCNU and acetaminophen killed 43% of the hepatocytes. Clearly, the potentiation by BCNU is not a simple consequence of the depletion of GSH.

Effect of BCNU and acetone on GSH metabolism. As will be recalled from Fig. 3, BCNU potentiated the toxicity of acetaminophen in the presence of 100 mM acetone. This effect was accompanied by a greater rate and extent of GSH depletion than that with acetaminophen and acetone alone. Fig. 5 details the time course of the cell killing (*left panel*) and the changes in GSH content (*right panel*) of hepatocytes that were treated with 5 mM acetaminophen and 100 mM acetone or with BCNU in addition to acetaminophen and acetone. In the presence of 100 mM acetone, BCNU potentiated both the rate and extent of the cell killing by 5 mM acetaminophen.

The control cells (Fig. 5, *right panel*) again displayed an increasing content of GSH over the 4 hr course of the experiment. The presence of 100 mM acetone and acetaminophen prevented the accumulation of GSH. There was no increase over the initial value in the GSH content of the hepatocytes during the same time period. By contrast, the addition of BCNU caused a loss of GSH from the hepatocytes treated with acetone and acetaminophen. Within 1 hr there was significantly less GSH in the cultures treated with BCNU as opposed to those treated with acetone and acetaminophen alone. In the presence of BCNU, the GSH content of the cells remained lower at 2

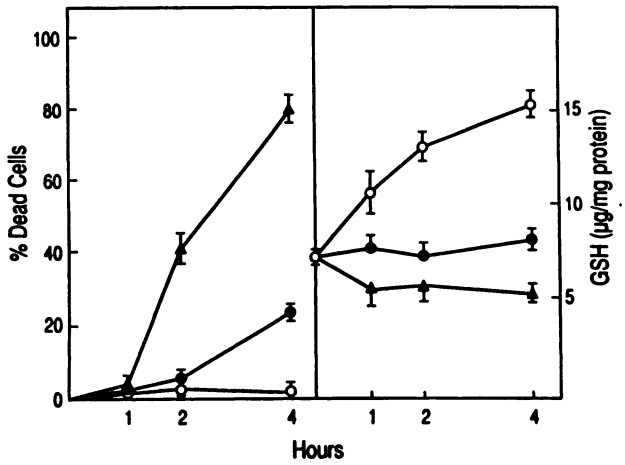

Fig. 5. Effect of BCNU and acetone on the time course of the changes in viability (*left*) and GSH metabolism (*right*) in hepatocytes treated with 5 mM acetaminophen. Hepatocytes cultured from the liver of a male rat pretreated with phenobarbital were left untreated (○) or given 5 mM acetaminophen and 100 mM acetone (●) or acetaminophen plus 100 mM acetone and 50 μM BCNU (▲). At the times indicated, the viability of the cells as well as their GSH content were measured. The results are the mean ± standard deviation of the determinations on three separate cultures.

TABLE 4

Protection by deferoxamine and DPPD in the presence or absence of acetone

Hepatocytes were isolated from male rats pretreated with three daily injections of 80 mg/kg of phenobarbital. After the cells were in culture for 3.5 hr they were treated with 5 mM acetaminophen and 50 μM BCNU either in the presence or absence of 100 mM acetone. DPPD (1 μM) was added simultaneously with the acetaminophen. Deferoxamine (20 mM) was added during the last hour of culture and then removed by washing before treatment with acetaminophen. Four hours after acetaminophen the viability of the cells was measured by the release of LDH into the culture medium. The results are the mean ± standard deviation of the determinations on three separate cultures.

Treatment	Dead Cells	
	−Acetone	+Acetone
	% of total	
1. Control	4 ± 1	3 ± 1
2. APAP[a] plus BCNU	50 ± 4	79 ± 6
3. APAP + BCNU + DPPD	4 ± 2	25 ± 4
4. APAP + BCNU + deferoxamine	5 ± 1	30 ± 3

[a] APAP, acetaminophen.

and 4 hr than in the hepatocytes that were given acetone and acetaminophen.

Prevention by deferoxamine and DPPD in the presence or absence of acetone. Table 4 details the protective effect of DPPD and deferoxamine against the cell killing by 5 mM acetaminophen and 50 μM BCNU. In the absence of acetone, 50% of the cells were killed by acetaminophen and BCNU (*line 2*). DPPD reduced the number of dead cells to that seen in control cultures (*line 3*). Similarly, the 1-hr pretreatment of the hepatocytes with 20 mM deferoxamine completely prevented the toxicity of acetaminophen (*line 4*). In the presence of acetone, 79% of the cells were killed by acetaminophen and BCNU. DPPD and deferoxamine reduced the cell killing to 25 and 30%, respectively, of the total hepatocytes.

BCNU potentiates the hepatotoxicity of acetaminophen in the intact animal. Table 5 documents the potentiation by BCNU of the hepatotoxicity of acetaminophen in the intact animal induced with 80 mg/kg of phenobarbital for 3 days. When such induced rats are treated with 750 mg/kg of

TABLE 5

Potentiation by BCNU of the toxicity of acetaminophen in the intact animal

All animals were male rats induced with 80 mg/kg of phenobarbital for 3 days and fasted overnight before use. BCNU was given 2 hr before acetaminophen, and all animals were sacrificed 6 hr later. The extent of liver necrosis was assayed by the content of serum glutamic oxaloacetic transaminase in the plasma. Results are the mean ± standard deviation of the determinations on three separate animals.

Treatment	Liver necrosis, SGOT[a]
	units/ml
1. Control	130 ± 9
2. BCNU (60 mg/kg)	150 ± 26
3. APAP[b] (750 mg/kg)	180 ± 16
4. BCNU and APAP	3550 ± 444

[a] SGOT, serum glutamic oxaloacetic transaminase.
[b] APAP, acetaminophen.

acetaminophen, there was no evidence of liver necrosis 6 hr later (*lines 1* and *3*). However, if BCNU was administered 2 hr before treating the animals with 750 mg/kg of acetaminophen, liver necrosis was readily detectable after 6 hr (*line 4*). BCNU alone had no effect on liver cell viability (*line 2*).

Discussion

The data presented in this report document that BCNU potentiates the hepatotoxicity of acetaminophen in both the phenobarbital-induced intact rat and in hepatocytes cultured from such animals. This conclusion is consistent with our previous demonstration that BCNU similarly potentiates the toxicity of acetaminophen when sensitivity to this hepatotoxin is induced with 3-methylcholanthrene (1, 2).

The present study was prompted by a recent report of an apparent inability to demonstrate a potentiation by BCNU of the killing by acetaminophen of isolated hepatocytes suspended in a medium containing 100 mM acetone (8). By contrast, the present study did observe potentiation by BCNU in the presence or absence of 100 mM acetone. The inability of the previous study to detect potentiation by BCNU can be readily explained by the obvious differences from the conditions obtaining in the present one.

The conditions used here to assess the effect of BCNU were such that acetaminophen in the presence of 100 mM acetone killed less than 50% of the hepatocytes by 4 hr (Fig. 3). Furthermore, with less than half of the hepatocytes dead from the combined effects of acetaminophen and acetone, there were still substantial GSH concentrations in remaining viable cells (Fig. 5). Thus, we could readily assess a potentiation by BCNU of either the rate or the extent of the cell killing, as well as evaluate an effect of BCNU on GSH metabolism. Indeed, BCNU potentiated both the rate and extent of cell killing with 5 mM acetaminophen and 100 mM acetone (Figs. 3 and 5), an effect that was accompanied by a greater rate and extent of GSH depletion (Fig. 5).

By contrast, Porubek *et al.* (8) killed 100% of the suspended hepatocytes with 5 mM acetaminophen and 100 mM acetone by 4 hr. Thus, the only effect of BCNU that could have been detected was an acceleration of the rate, rather than the extent of the cell killing. Whereas such an acceleration was not seen with acetaminophen, the cell killing by 3,5-dimethylacetaminophen, an analogue that cannot conjugate to glutathione upon oxidation to a quinone imine, was accelerated by 1–2 hr. The reason for the different result with 3,5-dimethylacetaminophen

as opposed to acetaminophen itself is readily deduced, without having to conclude that these compounds lethally injure hepatocytes by different mechanisms.

BCNU inactivates glutathione reductase, an effect that prevents the reduction of GSSG to GSH. The inhibition of glutathione redox cycling presumably leads to a faster rate of GSH depletion in cells that are intoxicated with acetaminophen. Such an accelerated rate of GSH depletion was indeed observed with 5 mM acetaminophen in the presence (Fig. 5) or absence (Fig. 4) of 100 mM acetone. However, it is important to emphasize that the critical factor in the potentiation by BCNU is the inability of GSH to redox cycle rather than the absolute extent of the GSH depletion. When GSH levels were depleted with 40 μM diethylmaleate to the same extent as occurred with 50 μM BCNU alone, there was no potentiation of the cell killing by acetaminophen (Table 2). This presumably reflects the fact that the residual glutathione content after treatment with diethylmaleate still allows GSH-GSSG redox cycling and, thus, continued detoxification of hydrogen peroxide. By contrast, the reduced GSH content in the presence of BCNU cannot participate in the detoxification of H_2O_2.

Interestingly, in the study of Porubek *et al.* (8) the rate and extent of GSH depletion with 3,5-dimethylacetaminophen was less than that with acetaminophen. Whereas acetaminophen depleted the suspended hepatocytes of over 80% of their initial content of GSH within 1 hr, 3,5-dimethylacetaminophen left greater than 40% of the initial content of GSH in the cells. Thus, BCNU could accelerate the cell killing by 3,5-dimethylacetaminophen, because there was still substantial GSH remaining in the hepatocytes. By contrast, the killing of 100% of the hepatocytes by 5 mM acetaminophen and 100 mM acetone was accompanied by such a severe depletion of GSH that there was probably little room left to see an effect of BCNU on the rate of cell killing. In other words, it is suspected that the extent of glutathione depletion in the absence of BCNU was such that GSH-GSSG redox cycling was already inhibited. Thus, inhibition of glutathione reductase by BCNU had no further effect. By contrast, the present study documents a potentiation by BCNU when less than 50% of the cells were killed by acetone and acetaminophen (Fig. 3). Under these conditions, there was no depletion of GSH with respect to the initial level (Fig. 5), and BCNU potentiated both the rate and the extent of the cell killing with 5 mM acetaminophen (Fig. 5). At the same time, BCNU accelerated the rate and extent of the depletion of GSH (Fig. 5).

An important conclusion emerges from this discussion. The discrepant results that distinguish our previous (1, 2) and present studies from that of Porubek *et al.* (8) cannot be attributed to the differences in the model systems used. The different results are clearly the consequence of the differing conditions to which these models were subjected in the course of the analyses reported. Furthermore, the present report documents that the conditions of the toxicity of acetaminophen that are obtained with hepatocytes prepared from animals induced with 3-methylcholanthrene are reproduced in the killing of hepatocytes from phenobarbital-induced rats. In particular, the potentiation by BCNU and the protection afforded by the chelation of a cellular pool of ferric iron indicate that oxidative mechanisms are involved in the killing by acetaminophen of cultured hepatocytes from animals induced with phenobarbital.

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