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KILLING OF CULTURED HEPATOCYTES BY THE MIXED-FUNCTION OXIDATION OF ETHOXYCOUMARIN*

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Abstract—Ethoxycoumarin is metabolized by mixed-function oxidation to give 7-hydroxycoumarin (umbelliferone) and acetaldehyde, without formation of an intermediate electrophile. Ethoxycoumarin was found, nevertheless, to injure cultured rat hepatocytes. Male hepatocytes were more sensitive than female to ethoxycoumarin. Phenobarbital increased cell killing, and SKF 525A, an inhibitor of ethoxycoumarin metabolism, prevented it. Neither umbelliferone nor acetaldehyde were toxic. Cellular glutathione decreased and oxidized glutathione (GSSG) accumulated in the culture medium. Sulfhydryl reagents prevented the cell killing without inhibiting metabolism. Lipid peroxidation was detected prior to evidence of cell death, and the antioxidant *N*,*N*′-diphenyl-phenylenediamine prevented both the lipid peroxidation and cell killing without inhibiting metabolism. Inhibition of glutathione reductase with 1,3-bis(chloroethyl)-1-nitrosourea potentiated the cell killing without increasing metabolism. Pretreatment of the cells with the ferric iron chelator deferoxamine reduced cell killing, again without inhibiting metabolism. Ferric chloride restored the sensitivity of deferoxamine-pretreated hepatocytes to ethoxycoumarin. These data define a new experimental model in which lethal liver cell injury is dependent on the metabolism of ethoxycoumarin but unrelated to its two known metabolites. An oxidative stress accompanying the cytochrome P-450-dependent metabolism of ethoxycoumarin is proposed as the mechanism coupling metabolism to lethal cell injury.

The study of such hepatotoxins as carbon tetrachloride [1], bromobenzene [2] and acetaminophen [2] has provided considerable insight into the mechanisms whereby chemicals can injure cells. A unifying concept derived from the early studies of toxic liver necrosis is the dependence of liver cell injury on the metabolism of the particular hepatotoxin. This conclusion served to focus subsequent studies on the mechanisms coupling metabolism of hepatotoxic chemicals to the consequent development of lethal cell injury.

The most commonly studied hepatotoxic chemicals are typically metabolized by cytochrome P-450-dependent mixed-function oxidation. For over a decade now, the mechanism generally assumed to couple mixed-function oxidation to liver cell injury has been the formation of an electrophilic and, therefore, reactive intermediate metabolite that can, in turn, interact with cellular macromolecules [2]. The covalent binding of such reactive electrophiles to critical cellular macromolecules has been invoked repeatedly to account for the liver cell injury resulting from the metabolism of a great many hepatotoxic chemicals as well as other chemicals with differing target cell specificities [3]. Such an hypothesis implies that the formation of reactive intermediates distinguishes the metabolism of those chemicals that are hepatotoxic from those that are metabolized and are not toxic.

The present report tests the implication of this hypothesis by examining the effects of ethoxycoumarin on cultured rat hepatocytes. Ethoxycoumarin is oxidatively de-ethylated to yield 7-hydroxycoumarin (umbelliferone) and acetaldehyde without formation of an intermediate electrophilic metabolite. Nevertheless, we found that ethoxycoumarin was toxic to cultured rat hepatocytes. Cell killing was dependent upon the metabolism of ethoxycoumarin, and we explored a number of ways whereby the extent of liver cell injury could be manipulated without accompanying changes in the extent of the metabolism of ethoxycoumarin.

MATERIALS AND METHODS

Sprague–Dawley rats (150–200 g) were obtained from the Charles River Breeding Laboratories, Inc. Male rats pretreated with phenobarbital were given sodium phenobarbital by intraperitoneal injections (80 mg/kg body wt/day) for 3 days. All animals were fasted overnight prior to use. Isolated hepatocytes, prepared as described previously [4], were plated in 25^2 cm flasks at a density of 1.6×10^6 cells per flask in Williams E medium (Grand Island Biological Co.) containing 10% heat-inactivated (56°, 30 min) fetal calf serum (Hazelton Dutchland Inc.), 10 I.U./ml penicillin, 10 μg/ml streptomycin and 0.02 units/ml

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insulin. After a 2-hr incubation (37°, 5% CO_2, 95% air), unattached dead cells and debris were removed by rinsing the cultures twice with prewarmed buffered balanced salt solution. The cultures were then incubated in complete Williams E medium with the additions as noted in the text. Cell killing was assayed by the release of lactic dehydrogenase or by the inclusion of trypan blue as previously described [4].

Ethoxycoumarin (Sigma), DPPD* (Eastman Kodak Co.), and umbelliferone (Sigma) were dissolved in dimethyl sulfoxide and added to the cultures at the times and concentrations noted in the text. Acetaldehyde (Sigma), SKF 525A (Smithkline Beckman Corp.) and ferric chloride (Fisher) were diluted in saline prior to their addition to the cultures. α-Mercaptopropionyl glycine was dissolved in 0.9% NaCl and added to the cultures at a final concentration of 2.5 mM. Deferoxamine mesylate (CIBA Pharmaceutical Co.) was dissolved in distilled water and added to the cultures where indicated between the first and second hour after placing the cells in culture and prior to washing the cells and exposing them to ethoxycoumarin. BCNU (1,3-bis[2-chloroethyl]-1-nitrosourea) was obtained from Bristol Laboratories and dissolved in dehydrated ethanol.

Glutathione reductase, glutathione peroxidase and catalase activities were measured as described previously [5]. The GSH content of the hepatocytes was determined by the method of Sedlak and Lindsay [6]. GSSG was measured fluorometrically according to Hissan and Hilf [7]. An aliquot of tissue culture medium was diluted with an equal volume of 20 mM EDTA. TCA (30 μl) was added to 500 μl of diluted medium. The samples were centrifuged at 10,000 *g* for 10 min at 4°. One hundred microliters of the supernatant fraction was incubated at room temperature with 100 μl of 0.04 *N*-ethylmaleimide for 30 min and the GSSG content was then measured. GSH in the culture medium was auto-oxidized in the absence of cells, and the amount of GSSG produced in this manner was subtracted from each of the values obtained in the presence of cells. Malondialdehyde was measured fluorometrically by an adaptation of the method of Yagi [8] as described elsewhere [4] and was standardized to the amount of hepatocyte protein initially present in each flask.

The metabolism of ethoxycoumarin was measured by quantitating the accumulation of the glucuronide and sulfate conjugates of umbelliferone by modification of the method of Ullrich and Weber [9]. At the times indicated, 0.5 ml of cell culture medium was removed and incubated anaerobically for 16–18 hr at 37° in 1 ml of acetate buffer (pH 4.5) containing 0.33 mg of β-glucuronidase (Sigma). An aliquot of the resulting mixture was then diluted in 0.25 M Hepes buffer (pH 7.5), and the fluorescence was measured (366 nm excitation, 450 nm emission). Standards were prepared by adding umbelliferone (7-OH-coumarin) to aliquots of complete Williams E medium. Preliminary studies evaluated the conjugation of umbelliferone added to hepatocyte cultures by measuring the difference in fluorescence before and after β-glucoronidase–sulfatase hydrolysis. Umbelliferone added to the culture medium was readily converted to non-fluorescent products. Treatment of the culture medium with glucuronidase and sulfatase restored much of the original fluorescence. Unconjugated umbelliferone was measured by the fluorescence remaining in the medium prior to treatment with glucuronidase/sulfatase. The increase in fluorescence after such treatment measured the glucuronides and sulfates. There was no remaining unconjugated umbelliferone after 24 hr with up to 100 μM ethoxycoumarin, and only 3% was unconjugated with 200 μM ethoxycoumarin. Between 70 and 80% of the conjugated umbelliferone represented glucuronides and sulfates.

* Abbreviations: DPPD, *N,N'*-diphenyl-phenylenediamine; GSSG, oxidized glutathione; GSH, reduced glutathione; LDH, lactic dehydrogenase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MDA, malondialdehyde; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid.

RESULTS

Ethoxycoumarin-induced cell killing. Freshly isolated hepatocytes, plated in plastic flasks and allowed to attach for 2 hr, were exposed to ethoxycoumarin. Few cells were killed with 100 μM ethoxycoumarin when hepatocytes were prepared from female rats (Table 1). Hepatocytes from male rats were more sensitive to ethoxycoumarin. After 24 hr, 35% of the cells were dead (Table 1). In turn, hepatocytes prepared from male rats pretreated for 3 days with phenobarbital (80 mg/kg body wt/day) were even more sensitive to ethoxycoumarin (Table 1). After 24 hr with 100 μM ethoxycoumarin, 65% of the hepatocytes were dead. All subsequent experiments employed cultured hepatocytes prepared from phenobarbital-induced male rats. Cell killing quantitated as the appearance of lactic dehydrogenase in the culture medium correlated closely with a second and independent assay of cell death, the uptake of trypan blue (data not shown).

Dependence of the cell killing on the metabolism of ethoxycoumarin. Ethoxycoumarin is metabolized to 7-hydroxycoumarin (umbelliferone) and acetaldehyde. Ethoxycoumarin metabolism was measured as the accumulation of the glucuronide and sulfate conjugation products of umbelliferone. Figure 1 illustrates both the time course of the metabolism of ethoxycoumarin (left panel) and the accompanying death of the hepatocytes (right panel) in the presence or absence of 10 μM SFK 525A, an inhibitor of mixed-function oxidation. Umbelliferone accumulation was evident within 1 hr and

Table 1. Ethoxycoumarin toxicity in hepatocyte primary cultures

Source of hepatocytes	Cell killing*
Phenobarbital-induced male rat	63 ± 5
Uninduced male rat	35 ± 1
Uninduced female rat	16 ± 1

Results represent the mean ± SD of determinations on three separate cultures.

* Percent dead cells 24 hr after treatment with 100 μM ethoxycoumarin.

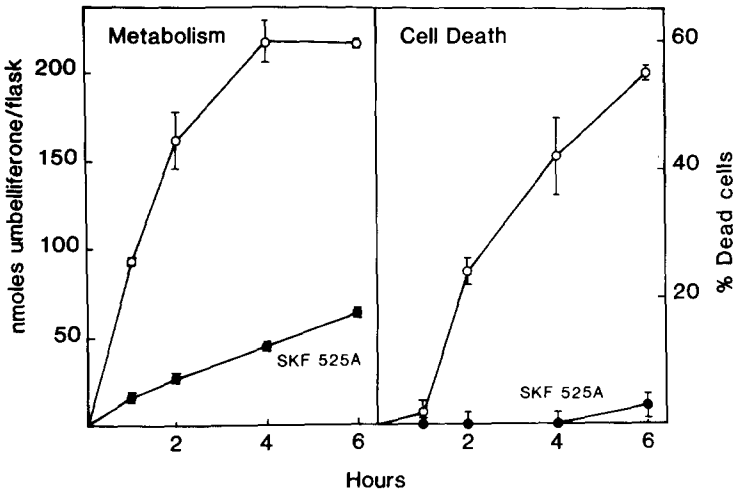

Fig. 1. Dependence on metabolism of the cell killing by ethoxycoumarin. Cultured hepatocytes were treated with 100 μm ethoxycoumarin in the presence (closed circles) or absence (open circles) of 10 μM SKF 525A. At the times indicated, the extent of the metabolism of ethoxycoumarin (left panel) and the associated cell killing (right panel) were measured. Results are the mean ± SD of the determinations on three separate cultures.

before there was any detectable cell killing. Umbelliferone formation continued to increase over the next 3 hr. By 6 hr approximately 25% of the ethoxycoumarin had been metabolized and 50–60% of the cells had been killed. SKF-525A inhibited the formation of umbelliferone, the metabolism of ethoxycoumarin was reduced by 80% over the 6 hr of the experiment, and the death of the hepatocytes was prevented almost completely (Fig. 1). This protection by SKF 525A was not a simple consequence of an inhibition of the uptake of ethoxycoumarin. SKF 525A had no effect on the conjugation of umbelliferone. In addition, the dependency on metabolism of the cell killing could not be attributed to the toxicity of either of the two known metabolites. Neither 200 μM umbelliferone nor acetaldehyde had any effect on the viability of the cultured hepatocytes (Table 2).

GSH metabolism in ethoxycoumarin-intoxicated hepatocytes. The metabolism of ethoxycoumarin was accompanied by a dose-dependent decrease in the GSH content of the hepatocytes (Table 3). This GSH depletion was not seen with either umbelliferone or acetaldehyde and was prevented by SKF 525A. A number of sulfhydryl reagents including β-mercaptoethanol, α-mercaptopropionyl glycine and *N*-acetylcysteine prevented the cell killing without inhibiting the metabolism of ethoxycoumarin. Figure 2 illustrates the effect of α-mercaptopropionyl glycine on the metabolism and toxicity of ethoxycoumarin. The number of dead cells was reduced from 60% to less than 20% without any change in the rate or extent of the formation of umbelliferone.

Table 2. Toxicity of ethoxycoumarin biotransformation products

	% Dead cells at 24 hr
Control	11 ± 4
200 μM Ethoxycoumarin	94 ± 5
200 μM Umbelliferone (7-hydroxycoumarin)	8 ± 3
200 μM Acetaldehyde	10 ± 1

Results are the mean ± SD of the determinations on three separate cultures.

Neither mercaptoethanol nor α-mercaptopropionyl glycine can be utilized for GSH synthesis. They cannot act, therefore, by simply maintaining GSH levels. Protection by sulfhydryl compounds against the liver necrosis produced by such hepatotoxins as bromobenzene and acetaminophen is generally attributed to the trapping of electrophilic metabolites that would otherwise interact with cellular constituents [2]. Such intermediates are not formed during the metabolism of ethoxycoumarin, and another explanation of the protective effect of sulfhydryls was needed. Sulfhydryl compounds are efficient protective agents against the toxicity of an acute oxidative stress, again without necessarily serving as precursors for the synthesis of GSH [10, 11].

The efflux and accumulation in the medium of GSSG have been used to quantitate acute oxidative stress in suspended rat hepatocytes. In particular, the cytochrome P-450 substrates ethylmorphine and benzphetamine increased GSSG efflux from hepatocyte suspensions prepared from phenobarbital-induced rats [12]. The accumulation of GSSG was presumed to result from the stimulation of hydrogen peroxide formation that can accompany mixed-function oxidation.

The metabolism of ethoxycoumarin was similarly accompanied by an accumulation of GSSG in the culture medium (Table 4). There was no GSSG detectable in the medium in the absence of ethoxycoumarin. After 1 hr in 100 μM ethoxycoumarin, there were 51.5 ± 2 nmoles GSSG in the medium. SKF 525A reduced the accumulation of GSSG to 11.5 ± 1 nmoles. GSSG accumulation was depen-

Table 3. Ethoxycoumarin-mediated depletion of GSH in the presence and absence of deferoxamine

Ethoxycoumarin (μM)	GSH (μg/mg protein) Minus deferoxamine	+20 mM Deferoxamine
0	6.88 ± 0.86	6.27 ± 0.15
25	5.53 ± 0.65	6.00 ± 0.56
50	4.57 ± 0.62	5.10 ± 0.10
100	3.52 ± 0.35	4.44 ± 0.39
200	3.30 ± 0.37	3.84 ± 0.55

Results are the mean ± SD of determinations on three separate cultures. Measurements were performed at the end of a 1-hr incubation before any loss of cell viability was detected.

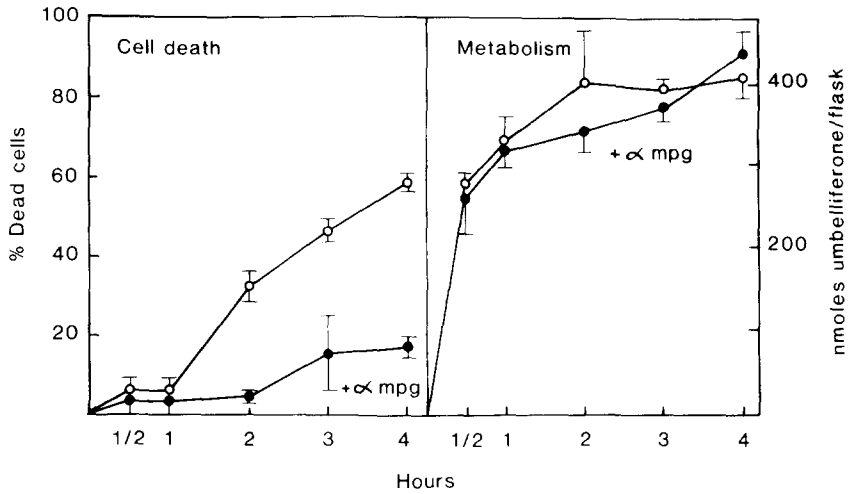

Fig. 2. Protection by the sulfhydryl reagent α-mercaptopropionyl glycine against the cell killing by ethoxycoumarin. Cultured hepatocytes were treated with 100 μM ethoxycoumarin in the presence or absence of 2.5 mM α-mercaptopropionyl glycine. At the times indicated, the extent of metabolism of ethoxycoumarin (right panel) and the associated cell killing (left panel) were measured. Results are the mean ±SD of the determinations on three separate cultures.

dent, therefore, on the metabolism of ethoxycoumarin. Figure 1 indicated that 80 ± 5 nmoles umbelliferone were formed during the first hour of exposure to 100 μM ethoxycoumarin. Every mole of H_2O_2 metabolized by glutathione peroxidase produces 1 mole of GSSG. The rate of H_2O_2 production, therefore, would seem to be approximately two-thirds the rate of metabolism of ethoxycoumarin. This is, of course, a minimum estimate of the rate of H_2O_2 formation, since H_2O_2 can also be catabolized by catalase and GSSG can be reduced to GSH by glutathione reductase. Table 3 indicated that the cells lost 3.4 μg GSH/mg protein after a 1-hr incubation with 100 μM ethoxycoumarin. Since there are about 3 mg total protein in each flask, the cells would have lost about 35 nmoles GSH. The greater accumulation of GSSG (50 nmoles GSSG requires 100 nmoles GSH) can be readily explained by the resynthesis of GSH.

Table 4. GSSG accumulation in hepatocytes treated with ethoxycoumarin

	GSSG (nmoles/flask)
Control cells (no ethoxycoumarin)	0
100 μM Ethoxycoumarin for 1 hr	51.5 ± 2.0
Ethoxycoumarin plus SKF 525A	11.5 ± 1.0

Results are the mean ± SD for determinations on three separate cultures.

Lipid peroxidation in ethoxycoumarin-intoxicated hepatocytes. Another consequence of acute oxidative stress is the peroxidation of cellular lipids. We have shown previously that the accumulation of malondialdehyde in the liver cell culture medium is a readily measured and sensitive index of the peroxidation of cellular lipids [4]. MDA could be detected in the cultures within 30 min after exposure to ethoxycoumarin and before any loss of viability of the hepatocytes (Table 5). The content of MDA continued to increase over the next half-hour, again in the absence of cell killing. By 1.5 hr the content of MDA had increased even further, and there were now dead cells in the cultures. As the content of malondialdehyde continued to increase in the cultures, the extent of cell killing also increased. Figure 3 illustrates the correlation between ethoxycoumarin concentration, malondialdehyde production and cell death.

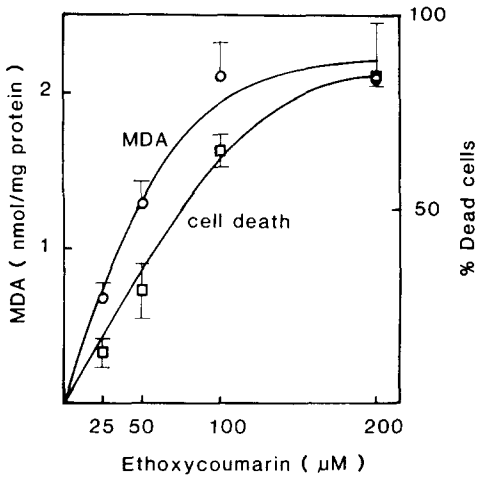

Fig. 3. Correlation between ethoxycoumarin concentration, cell killing and malondialdehyde formation. Cultured hepatocytes were treated with the concentrations of ethoxycoumarin shown in the figure. After 4 hr, the extent of cell killing (squares) and the accumulation of MDA in the separate flasks were measured. Results are the mean ±SD of the determinations on three separate cultures.

Table 5. Ethoxycoumarin-mediated cell killing and malondialdehyde formation in hepatocyte primary cultures

Time (min)	MDA (nmoles/mg protein)	Cell death (%)
0	0.10 ± 0.03	0
15	0.13 ± 0.02	0
30	0.23 ± 0.01	0
60	0.42 ± 0.01	2 ± 1
90	0.92 ± 0.04	20 ± 3
120	1.63 ± 0.17	43 ± 2
120 + DPPD	0.12 ± 0.03	0
120 control	0.09 ± 0.02	0

Results are mean ± SD of determinations on three separate cultures.

Addition of the antioxidant *N,N*-diphenyl-*p*-phenylenediamine to the cultures prevented both the peroxidation of cellular lipids (Table 5) and the killing of the hepatocytes (Table 5 and Fig. 4). Prevention of lipid peroxidation was not simply a consequence of an inhibition of the metabolism of ethoxycoumarin. Figure 4 shows that DPPD reduced the extent of cell killing by ethoxycoumarin (left panel) without altering the metabolism of ethoxycoumarin (right panel). In fact, the protective action of DPPD was actually associated with enhanced metabolism, presumably a reflection of a healthier cell population.

Potentiation of ethoxycoumarin toxicity by inhibition of glutathione reductase. The glutathione redox cycle of the hepatocyte plays an important role in the defense against an oxidative stress [4, 10, 11, 13]. Selective inactivation of glutathione reductase by BCNU sensitized suspensions [4, 11] or cultures [10] of hepatocytes to adriamycin [4], *t*-butyl-hydroperoxide [11], menadione [13] or to hydrogen peroxide formed by glucose oxidase [10].

Figure 5 shows that BCNU similarly potentiated the toxicity of ethoxycoumarin. Cultured hepatocytes were treated with increasing concentrations of ethoxycoumarin in the presence or absence of 25 μM BCNU. The extent of cell killing was measured after 4 hr. BCNU alone had no effect on the viability of the cells. In the absence of BCNU, there was little cell killing with up to 50 μM ethoxycoumarin, whereas 100 μM killed 25–30% of the cells. In the presence of BCNU, 25 μM ethoxycoumarin killed 30–40% of the cells, 50 μM killed 50–60%, and 100 μM killed over 70% of the cells. This increased cell killing with BCNU was still dependent upon the metabolism of ethoxycoumarin. SKF 525A prevented the cell killing in those cultures treated with BCNU and ethoxycoumarin (Fig. 5). Sulfhydryl reagents effectively protected the hepatocytes in the presence of both BCNU and ethoxycoumarin. Con-

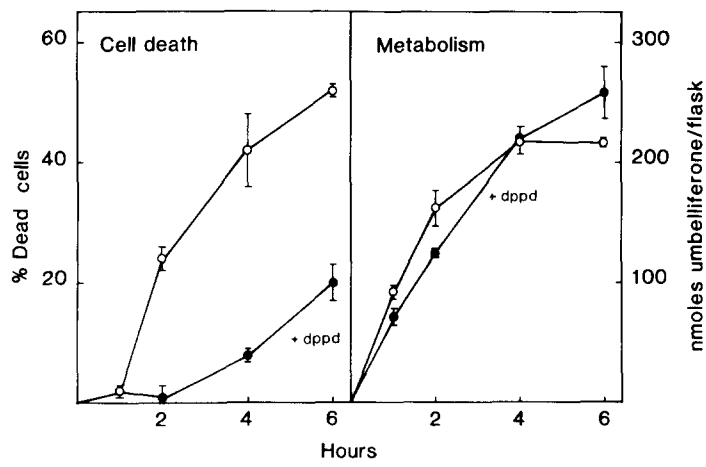

Fig. 4 Prevention by DPPD of the killing of cultured hepatocytes by ethoxycoumarin. Cultured hepatocytes were treated with 100 μM ethoxycoumarin in the presence (closed circles) or absence (open circles) of 2 μM DPPD. At the times indicated, the extent of the metabolism of ethoxycoumarin (right panel) and the associated cell killing (left panel) were measured. Results are the mean ±SD of the determinations on three separate cultures.

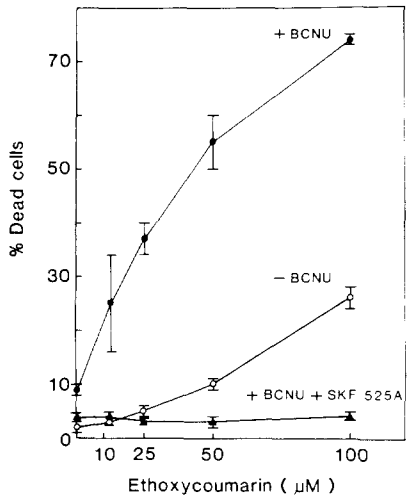

Fig. 5. Potentiation by BCNU of the toxicity of ethoxycoumarin. Cultured hepatocytes were treated with increasing doses of ethoxycoumarin in the presence or absence of 25 μM BCNU. Where indicated 10 μM SKF 525A was added to those cultures treated with both BCNU and ethoxycoumarin. After 4 hr, the viability of the cells was measured by the extent of release of LDH into the culture medium. Results are the mean ± SD of the determinations on three separate cultures.

sistent with previous data [4], 25 μM BCNU inhibited glutathione reductase by 75% without affecting glutathione peroxidase or catalase. SKF 525A had no effect on the inhibition of glutathione reductase produced by 25 μM BCNU.

The enhanced cell killing in the presence of BCNU occurred without any change in the metabolism of ethoxycoumarin. Figure 6 compares the metabolism (right panel) and the cell death (left panel) in hepatocyte cultures treated with 50 μM ethoxycoumarin in the presence and absence of 25 μM BCNU. There was little cell killing detected at 4 hr in the absence of BCNU. With BCNU, however, 60% of the cells died. The metabolism of ethoxycoumarin during the first hour was identical with or without BCNU. In the absence of BCNU, there was significantly more metabolism of ethoxycoumarin between 1 and 4 hr. At 4 hr and without BCNU, 142 nmoles of umbelliferone were present in the cultures and 10% of the cells were dead. In contrast, 108 nmoles of umbelliferone were formed in BCNU-treated cultures and 60% of the cells were killed. A reduced metabolism of ethoxycoumarin with BCNU is again attributable to the enhanced cell killing.

Dependency of ethoxycoumarin toxicity on intracellular iron. In a preliminary experiment, freshly isolated hepatocytes were placed in culture for 1 hr and then exposed to concentrations of the ferric iron chelator deferoxamine from 0.5 to 20 mM for an additional hour. The cultures were washed and then treated with 200 μM ethoxycoumarin for 4 hr. Increasing protection was seen with increasing deferoxamine. Figure 7 illustrates the effect of a 1-hr pretreatment with 20 mM deferoxamine on the time course of the metabolism (right panel) of 200 mM ethoxycoumarin and the accompanying cell killing (left panel). Pretreatment with deferoxamine was without effect on the metabolism of ethoxycoumarin for the first 4 hr. Between 4 and 6 hr, more ethoxycoumarin was metabolized by deferoxamine-pretreated cells. At the same time, deferoxamine reduced the cell killing, particularly during the first 4 hr. Between 4 and 6 hr, there was an increase by about 20% in the number of dead cells in the deferoxamine-treated cultures. Since deferoxamine did not inhibit the metabolism of ethoxycoumarin, there was no effect of the deferoxamine pretreatment on the decrease in cellular GSH. The hepatocyte content of GSH declined with increasing concentrations of ethoxycoumarin to the same extent with or without the deferoxamine pretreatment (Table 3).

Deferoxamine pretreatment reduced the cell killing by ethoxycoumarin in BCNU-treated cultures

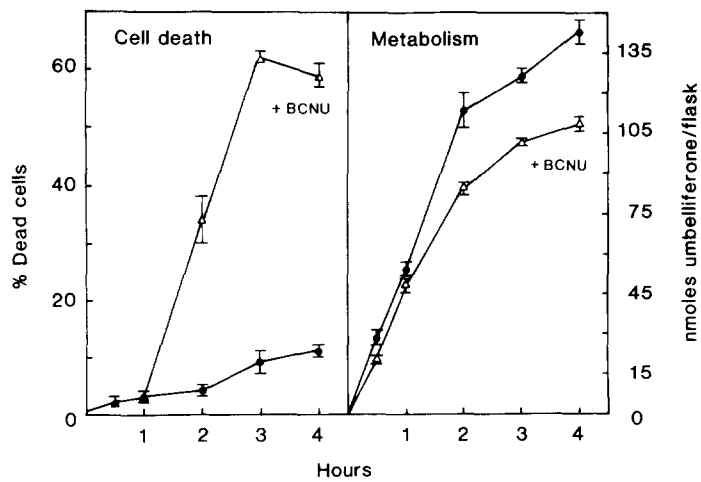

Fig. 6. Effect of BCNU on the time course of the metabolism and toxicity of ethoxycoumarin. Cultured hepatocytes were treated with 50 μM ethoxycoumarin in the presence or absence of 25 μM BCNU. At the time indicated, the extent of the metabolism of ethoxycoumarin and the associated cell killing were measured. Results are the mean ± SD of the determinations on three separate cultures.

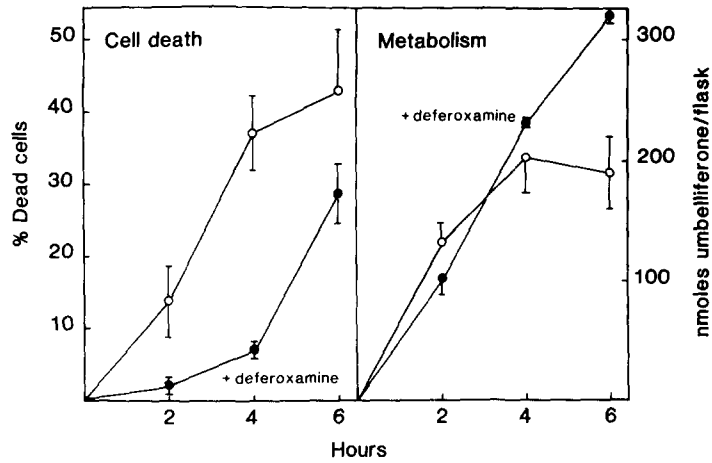

Fig. 7. Prevention by deferoxamine of the toxicity of ethoxycoumarin. Cultured hepatocytes were pretreated for 1 hr with 20 mM deferoxamine. After washing the cultures, the cells were treated with 100 μM ethoxycoumarin. At the times indicated, the extent of the metabolism of ethoxycoumarin (right panel) and the associated cell killing (left panel) were measured in the deferoxamine-pretreated (closed circle) and control cultures (open circles). Results are the mean ± SD of the determinations on three separate cultures.

(Table 6). This protective effect of deferoxamine was again achieved without inhibition of the metabolism of ethoxycoumarin. In fact, the deferoxamine-pretreated cells actually produced more umbelliferone than those given BCNU.

The sensitivity of deferoxamine-pretreated hepatocytes to ethoxycoumarin, in the presence or absence of BCNU, was restored by the addition of 1 mM $FeCl_3$ to the cultures. This effect of $FeCl_3$ was not dependent upon an increase in the metabolism of ethoxycoumarin. As shown in Table 6, there was actually less total umbelliferone formed in the iron-treated cultures, again most likely as a result of the greater cell injury in these cultures. There was no effect of 1 mM $FeCl_3$ alone on the viability of the cultured hepatocytes over the 4 hr indicated in Table 6.

DISCUSSION

Ethoxycoumarin is metabolized by cytochrome P-450-dependent mixed-function oxidation to yield umbelliferone and acetaldehyde without formation of an intermediate electrophile. Ethoxycoumarin would not be expected to be toxic, if mixed-function oxidation were coupled to cell injury by the formation of reactive electrophiles that can covalently bind to vital macromolecules. The present study documents, however, that ethoxycoumarin can indeed kill cultured rat hepatocytes.

The toxicity of ethoxycoumarin depends on metabolism. Hepatocytes prepared from male rats were more sensitive to ethoxycoumarin than were cells from females (Table 1). Induction of cytochrome P-450 by phenobarbital further increased the sensitivity of male hepatocytes (Table 1). Inhibition of the metabolism of ethoxycoumarin with SKF 525A reduced the cell injury (Fig. 1). Neither of the two known metabolites of ethoxycoumarin, umbelliferone or acetaldehyde, was capable of injuring the hepatocytes (Table 2). We conclude that the metabolism of ethoxycoumarin generates an agent, other than the two known metabolites, that is capable of lethally injuring the hepatocytes. Clues to the

Table 6. Protective effect of deferoxamine against ethoxycoumarin-mediated cell killing and its reversal by iron*

	Ethoxycoumarin concentration			
	50 μM		100 μM	
	% Dead cells	Metabolism	% Dead cells	Metabolism
BCNU	59 ± 3	56.5 ± 5.3†	63 ± 4	74.9 ± 6.1
BCNU + deferoxamine	20 ± 6	65.7 ± 9.3	36 ± 4	93.7 ± 4.5
BCNU + deferoxamine + $FeCl_3$	33 ± 4	53.0 ± 6.0	53 ± 1	90.2 ± 5.0

* Results are the mean ± SD of the cell killing at the end of a 4-hr incubation.

† Results are the mean ± SD of nmoles umbelliferone accumulated per flask during the course of a 4-hr incubation with ethoxycoumarin.

mechanism of cell injury are provided by the ways that the toxicity of ethoxycoumarin could be manipulated.

Conditions were presented in which the toxicity of ethoxycoumarin varied without corresponding changes in the extent of metabolism. Sulfhydryl reagents (Fig. 2), the antioxidant DPPD (Fig. 4), and the ferric iron chelator deferoxamine (Fig. 7) reduced the extent of cell killing without reducing ethoxycoumarin metabolism. Inhibition of glutathione reductase with BCNU enhanced the toxicity of ethoxycoumarin without increasing metabolism (Figs. 5 and 6).

Lipid peroxidation accompanied the cell killing by ethoxycoumarin (Table 5). The antioxidant DPPD prevented the peroxidation of cellular lipids (Table 5) and prevented the cell killing (Fig. 4). The peroxidation of lipids in ethoxycoumarin-intoxicated hepatocytes could simply represent its occurrence in already dead or dying cells. This would imply that there is no causal relationship between lipid peroxidation and cell killing. Against this explanation are two arguments. First, lipid peroxidation preceded the appearance of LDH in the culture medium (Table 5). Second, DPPD prevented both the cell killing and the lipid peroxidation. Lipid peroxidation alone would be affected if it were simply occurring in dead or dying cells. On the other hand, both the lipid peroxidation and the cell killing would be prevented by DPPD if DPPD simply inhibited the metabolism of ethoxycoumarin. In such a case, lipid peroxidation would again bear no necessary causal relationship to the hepatocyte injury. DPPD did not, however, inhibit the metabolism of ethoxycoumarin (Fig. 4). Two alternative explanations remain. On the one hand, the metabolism of ethoxycoumarin generates a mechanism that induces both lipid peroxidation and independent lethal injury. DPPD interferes with this mechanism, thereby preventing both cell killing and lipid peroxidation. On the other hand, the metabolism of ethoxycoumarin generates an agent that induces lipid peroxidation which, in turn, lethally injures the cells. DPPD prevents cell killing by preventing lipid peroxidation. Several reasons make an acute oxidative stress the most likely mechanism coupling ethoxycoumarin metabolism to the resulting cell injury. Only the second alternative above is consistent with the lethal mechanism being an acute oxidative stress. DPPD has no effect on an acute oxidative stress other than to prevent the appearance of lipid peroxidation [10].

The accumulation of GSSG in the culture medium indicates that the metabolism of ethoxycoumarin imposes an oxidative stress on the cells. GSH oxidation to GSSG accompanies the reduction of H_2O_2 and other peroxides by glutathione peroxidase. The stoichiometry of GSSG accumulation agreed closely with the stoichiometry of both the metabolism of ethoxycoumarin and the depletion of GSH.

Potentiation of the toxicity of ethoxycoumarin by BCNU closely resembles the similar effect of BCNU on the susceptibility of hepatocytes to adriamycin [4], menadione [13] or hydrogen peroxide itself [10]. With adriamycin and menadione, it is presumed that a one-electron reduced semiquinone-like metabolite is auto-oxidized to form superoxide anions that dismutate to give hydrogen peroxide [11, 14]. Incubation of BCNU-treated hepatocytes with adriamycin [4] or menadione [13] decrease cell viability with an accompanying increase in lipid peroxidation [4]. Vitamin E [4] or DPPD [10] protect from both the peroxidation of lipids and the cell killing. In the present study, BCNU similarly increased the toxicity of ethoxycoumarin (Fig. 5). This potentiation by BCNU depended upon the metabolism of ethoxycoumarin (Fig. 5), but could not be explained by changes in the rate or extent of metabolism. BCNU would seem, therefore, to make hepatocytes more sensitive to the mechanism that couples metabolism to cell injury. That this mechanism is an oxidative stress is suggested by (1) the fact that there is evidence of such stress in the accumulation of GSSG and (2) the fact that BCNU sensitizes hepatocytes to activated oxygen species.

We have shown recently that the toxicity of hydrogen peroxide is dependent upon a cellular source of ferric iron [15]. Cultured hepatocytes pretreated with the ferric iron chelator deferoxamine were resistant to the toxicity of H_2O_2 generated either by glucose oxidase or by the metabolism of menadione. Similarly, it has been shown that 1,10-phenanthroline, another iron chelator, prevents the killing of 3T3 cells by H_2O_2 [16].

Pretreatment of the hepatocytes with deferoxamine reduced the cell killing by ethoxycoumarin (Fig. 7 and Table 6). A number of interpretations of the action of deferoxamine can be entertained. Conceivably the ferric iron in cytochrome P-450 was affected with resultant reduction in the metabolism of ethoxycoumarin. Pretreatment of the hepatocytes with 20 mM deferoxamine, however, had no effect on the metabolism of ethoxycoumarin at the same time that it reduced the associated cell killing (Fig. 7). Alternatively, it might be argued that the effect of deferoxamine was to chelate iron present in the tissue culture medium. The pretreatment regimen adopted makes such an explanation also unlikely. The effect of deferoxamine had to be on the hepatocytes themselves. Deferoxamine was not present when the cells were treated subsequently with ethoxycoumarin. Finally, it might be held that the effect of deferoxamine has nothing necessarily to do with its action as an iron chelator, but rather with some as yet unknown action that prevents the cell killing. Such an argument is countered by the fact that addition of $FeCl_3$ to the cultured medium restored the sensitivity of deferoxamine-pretreated hepatocytes to ethoxycoumarin (Table 6).

In summary, with the exception of SKF 525A, each of the ways that were found to modify the cell killing by ethoxycoumarin can be related to a similar change in the extent of the cell injury accompanying oxidative stress. One can envision, therefore, a sequence in which the metabolism of ethoxycoumarin imposes an oxidative stress on the hepatocyte most likely as a result of the formation of hydrogen peroxide accompanying the cycling of the cytochrome P-450 system. Lipid peroxidation is, in turn, induced; loss of membrane integrity with resultant liver cell death follows. Such a scheme provides an alternative to the covalent binding of reactive

metabolites as the mechanism coupling mixed-function oxidation to irreversible liver cell injury.

REFERENCES

1. R. O. Recknagel and E. A. Glende, Jr., *CRC Crit. Rev. Toxic.* **2**, 263 (1973).
2. J. R. Gillette, J. R. Mitchell and G. G. Brodie, *A. Rev. Pharmac.* **14**, 271 (1974).
3. H. J. Zimmerman, *Hepatotoxicity: The Adverse Effects of Drugs and Other Chemicals on the Liver.* Appleton-Century-Crofts, New York (1978).
4. A. Casini, M. Giorli, R. J. Hyland, A. Serroni, D. Gilfor and J. L. Farber, *J. biol. Chem.* **257**, 6721 (1982).
5. J. R. Babson, N. S. Abell and D. J. Reed, *Biochem. Pharmac.* **30**, 2299 (1981).
6. J. Sedlak and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
7. P. J. Hissan and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
8. K. Yagi, *Biochem. Med.* **15**, 212 (1976).
9. V. Ullrich and P. Weber, *Hoppe-Seyler's Z. physiol. Chem.* **353**, 1171 (1972).
10. P. E. Starke and J. L. Farber, *J. biol. Chem.* **260**, 86 (1985).
11. S. Bellomo, S. A. Jewell, H. Thor and S. Orrenius, *Proc. natn. Acad. Sci. U.S.A.* **79**, 6842 (1982).
12. D. P. Jones, L. Eklow, H. Thor and S. Orrenius, *Archs Biochem. Biophys.* **210**, 505 (1981).
13. H. Thor, M. T. Smith, P. Hartzell, G. Bellomo, S. A. Jewell and S. Orrenius, *J. biol. Chem.* **257**, 12419 (1982).
14. N. R. Nachur, S. L. Gordon, M. V. Gee and K. Hidea, *Proc. natn. Acad. Sci. U.S.A.* **76**, 954 (1979).
15. P. E. Starke and J. L. Farber, *J. biol. Chem.* **260**, 10099 (1985).
16. A. C. Mello Fillo, M. E. Hoffmann and R. Meneghini, *Biochem. J.* **218**, 273 (1984).