

Liposome-encapsulated Superoxide Dismutase Prevents Liver Necrosis Induced by Acetaminophen

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Liposome-encapsulated human recombinant superoxide dismutase (LSOD) protected male rats that were pretreated with 3-methylcholanthrene from the liver necrosis produced by acetaminophen. By contrast, SOD-free liposomes, free SOD, or heat-denatured LSOD had no protective effect. Liposome-encapsulated SOD did not simply delay the onset of liver necrosis. A second dose of LSOD at 12 hours prevented the necrosis of the liver as assessed 24 hours after treatment with 500 mg/kg body weight of acetaminophen. Liposome-encapsulated human recombinant superoxide dismutase did not alter the metabolism of acetaminophen as assessed by either the rate or extent of the depletion of hepatic stores of glutathione or by the extent of the covalent binding of the metabolites of [³H]acetaminophen to total liver cell proteins. Evidence of the peroxidation of lipids in the accumulation of malondialdehyde in the livers was detected within 3 hours of the administration of acetaminophen and before the appearance of liver necrosis. Liposome-encapsulated human recombinant superoxide dismutase prevented the accumulation of malondialdehyde in parallel with the prevention of liver necrosis. Finally, LSOD also prevented the potentiation by 1,3-bis(2-chloroethyl)-1-nitrosourea of the hepatotoxicity of acetaminophen. These data document the participation of superoxide anions in the hepatotoxicity of acetaminophen in intact rats. (Am J Pathol 1990, 136:787-795)

The killing of rodent liver cells by acetaminophen, a widely used analgesic, has been an important model in defining

the mechanisms by which drugs and other chemicals produce lethal cell injury. Liver cell necrosis results from the metabolism of acetaminophen by cytochrome P450-dependent mixed-function oxidation. Whereas there is general agreement that *N*-acetyl-*p*-benzoquinone imine (NAPQI) is the reactive metabolite of the biotransformation of acetaminophen,^{1,2} the precise mechanism of its formation is not defined, and the mechanism by which the metabolism of acetaminophen leads to the necrosis of hepatocytes is a matter of continuing study.

Based on studies in the early 1970s,³⁻⁷ it has been widely held that the arylation of critical hepatic macromolecules by the reactive metabolite of acetaminophen, namely NAPQI, mediates the liver necrosis. By contrast, more recent studies with intact mice⁸⁻¹⁰ or cultured rat hepatocytes¹¹⁻¹⁴ suggest a role for activated oxygen species in the hepatotoxicity of acetaminophen.

The sensitivity of cultured rat hepatocytes to acetaminophen is induced by pretreatment of the animals with 3-methylcholanthrene. Inhibition of glutathione reductase by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), previously shown to sensitize hepatocytes to an oxidative stress,¹⁵⁻¹⁷ potentiates the toxicity of acetaminophen without increasing the covalent binding of acetaminophen metabolites.¹¹ Superoxide dismutase and catalase prevent the cell killing.¹² Pretreatment of the hepatocytes with the ferric iron chelator deferoxamine, known to reduce the sensitivity of hepatocytes to an oxidative stress,¹⁶ prevents the cell killing without reducing covalent binding.¹¹ These data document the participation of oxygen radicals in the killing of cultured hepatocytes by acetaminophen in this model. Furthermore, they suggest that hydroxyl radicals generated by an iron-catalyzed Haber Weiss reaction mediate the cell injury. Specifically, superoxide anions re-

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duce an endogenous pool of ferric to ferrous iron. The latter reacts with hydrogen peroxide to generate the hydroxyl radical. Thus, superoxide dismutase prevents the formation of hydroxyl radicals by inhibiting the formation of ferrous iron needed for the Fenton reaction. In turn, catalase prevents the formation of hydroxyl radicals by removing hydrogen peroxide.

The present report was prompted by a need to confirm that these recent insights into the action of acetaminophen are relevant to the pathogenesis of liver necrosis in the intact animal. The data show that the hepatotoxicity of acetaminophen can be prevented by treatment with liposome-encapsulated superoxide dismutase. Thus, a critical feature of the killing of cultured hepatocytes by acetaminophen has been reproduced in the intact animals with the demonstration of the participation of superoxide anions in the genesis of liver cell necrosis.

Materials And Methods

Animals

Male Sprague-Dawley rats (150 g) were obtained from Charles River Japan Inc., (Atsugi, Kanagawa, Japan). The animals were fed *ad libitum* for 1 week before use (Oriental MF Pellet Diet; Oriental Yeast Co. Ltd., Itabashi, Tokyo, Japan), and kept in stainless steel, wired cages in an air-conditioned room at 25°C with a 12-hour light-dark cycle. All animals received 25 mg/kg of body weight of 3-methylcholanthrene (Sigma Chemical Co., St. Louis, MO) by intraperitoneal injection of a 10 mg/ml solution in corn oil 18 hours before use and then fasted overnight.

Reagents

Acetaminophen (APAP) (Sigma) was administered by intraperitoneal injection of a 250 mg/ml solution in dimethyl sulfoxide (Sigma). The BCNU (Bristol Laboratories, Syracuse, New York) was administered by intraperitoneal injection of 60 mg/kg of body weight as a 30-mg/ml solution in dehydrated ethanol 2 hours before treatment of the rats with APAP. Human recombinant Cu-Zn superoxide dismutase (SOD) (4000 U/mg of protein by the nitroblue tetrazolium assay) was a gift of the Toyo Jozo Co. Ltd., Ohito, Shizuoka, Japan. The heat inactivation of SOD (confirmed by enzyme assay) was accomplished in an autoclave at 110°C for 20 minutes. Superoxide dismutase was encapsulated within liposomes by the method of Michelson et al.¹⁹ In brief, phosphatidylcholine, cholesterol, and stearylamine (5:1:1) were dissolved in diethyl ether and evaporated to form a thin layer. Superoxide dismutase or heat-inactivated SOD was dissolved in 100 mmol/l

sodium phosphate buffer, pH 7.0, containing 5% glucose and added to the layered lipids. The sample then was heated to 60°C, vortexed, and sonicated in the water bath for 30 minutes. The resulting liposomes had an average diameter of 500 nm and were collected by chromatography on Sephadex G-50 (Pharmacia LKB Biotechnology Group, Shimagawa, Tokyo, Japan) and centrifugation for 1 hour at 20,000g. The resulting liposomes (LSOD) contained 571.4 U of SOD (0.143 mg of protein) per milligram of liposome lipid. Liposome-encapsulated heat-denatured SOD (LSODden) contained 0.143 mg of protein per milligram of liposome lipid. The SOD-free liposomes (56 mg of lipid/ml), LSOD (32,000 U/ml), free SOD (FSOD, 32,000 U/ml), and LSODden (8 mg of protein/ml) all were administered at the doses indicated in the text by intraperitoneal injection as solutions or suspensions in a buffer containing 100 mmol/l sodium phosphate, pH 7.0, and 5% glucose.

Assessment of Liver Necrosis

Liver cell necrosis was quantitated by determination of the activities in the serum of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). The animals were anesthetized with ethyl ether. Blood was removed from the aorta at its bifurcation, allowed to clot at room temperature, and centrifuged for 20 minutes at 3000g at 25°C. The serum was assayed for AST and ALT as previously described²⁰ and for ALP according to Bowers and McComb.²¹ After removal of blood, the livers were excised, blotted, and used for histologic examination or as described below.

Covalent Binding

The covalent binding of the metabolites of [³H]acetaminophen (*p*-[³H(G)]-hydroxyacetanilide, 9.9 Ci/mmol, New England Nuclear Corp., Boston, MA, diluted with unlabeled APAP to 0.5 mCi/mmol and 250 mg/ml, administered by intraperitoneal injection at a dose of 500 mg/kg of body weight) was determined by modification of the method of Jollow et al.²² A 1-g sample of the liver or medial femoral muscle was homogenized in 3 ml of ice-cold 50 mmol/l sodium phosphate buffer, pH 7.4, using an Ultra-Turrax homogenizer (Junke and Kunkel KG, Staufen, West Germany). One milliliter of the homogenate was mixed with 2 ml of ice-cold 10% trichloroacetic acid (TCA; 100% solution from Wako Pure Chemical Industries, Osaka, Japan) and sonicated on ice by using a Microson Ultrasonic Cell Disruptor (Heat Systems-Ultrasonics Inc., Farmingdale, NY) at its maximal output for 15 minutes with intermittent interruptions. After centrifugation of the soni-

cate for 10 minutes at 10,000g at 4°C, the pellet was taken and prepared according to Rao and Recknagel.²³ The dry protein residue was dissolved in 4.5 ml of 1 N NaOH at 50°C. A 0.1-ml aliquot was mixed with 10 ml Ultra-Gel II (Nacalai Tesque Inc., Kyoto, Japan) and counted in a scintillation spectrometer. The protein content of the NaOH solution was measured using the BCA Protein Assay employing bicinchoninic acid (Pierce Chemical Co., Rockford, IL).

Measurement of Glutathione

Hepatic reduced glutathione was measured by modification of the method of Sedlak and Lindsay.²⁴ Disodium ethylenediamine tetra-acetate (EDTA), tris(hydroxymethyl)-aminomethane (TRIZMA base), and reduced glutathione (GSH) were from Sigma. A 200-mg sample of liver was homogenized in 8 ml of ice-cold 20 mmol/l EDTA using an Ultra-Turrax homogenizer. Five milliliters of the homogenate or a standard solution was added to 4 ml of H₂O and 1 ml of 50% TCA and sonicated as above. After centrifugation of the sonicate for 15 minutes at 3000g, a 1-ml aliquot of the supernatant was mixed with 2 ml of 400 mmol/l Tris-HCl buffer, pH 8.9, containing 20 mmol/l EDTA and 0.05 ml of 10 mmol/l 5,5'-dithiobis-(2-nitro-benzoic acid) (Sigma). The resulting absorbance at 412 nm was then measured. The protein content of the homogenate was measured with the BCA Protein Assay.

Measurement of Lipid Peroxidation

Hepatic lipid peroxidation was quantitated as the accumulation of malondialdehyde (MDA) by modification of the method of Yagi.²⁵ A 500-mg piece of liver was homogenized in 4.5 ml of ice-cold 50 mmol/l sodium phosphate buffer, pH 7.4, using the Ultra-Turrax homogenizer. After adding 0.5 ml of ice-cold 50% TCA, the sample was sonicated and centrifuged for 20 minutes at 20,000g at 4°C. Two milliliters of the supernatant were mixed with 2 ml of 0.67% 2-thiobarbituric acid (Sigma) and boiled for 10 minutes. After cooling on ice, 1.6 ml of *n*-butanol were added and the solution was centrifuged for 15 minutes at 3000g at 25°C. The fluorescence of the supernatant was determined at 515 nm excitation and 553 nm emission. Standards were prepared by diluting malondialdehyde bis(dimethyl acetal) (Aldrich Chemical Co. Inc., Milwaukee, WI) 1:1000 with methanol. The resulting solution was mixed 1:1 with 0.2 N HCl, left overnight at room temperature, and 1.65 ml was added to 8.35 ml of methanol. Appropriate aliquots of this solution, containing 1.25 to 5 nmol of MDA, were added to 4.5 ml of the phosphate

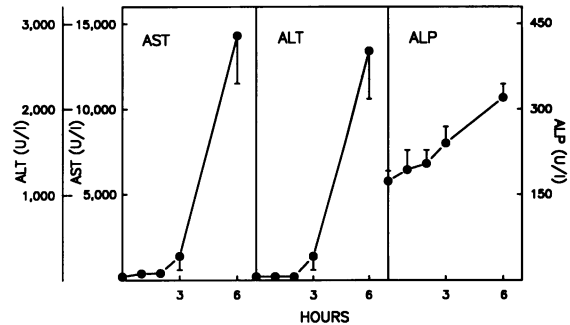


Figure 1. Time course of induction of liver cell necrosis by 500 mg/kg body weight of acetaminophen. The results are the mean \pm SD of the determinations on four or five animals. The 3-hour values for AST, ALT, and ALP were significantly different from the "0" time value at $P < 0.05$.

buffer used to homogenize the livers and then processed in a manner similar to these samples.

The statistical significance of the data was determined by the paired Student's *t*-test.

Results

Acetaminophen-induced Liver Cell Necrosis

Fasted male rats induced with 3-methylcholanthrene were treated with 500 mg/kg of body weight of acetaminophen. Figure 1 illustrates the time course of the resulting liver cell necrosis as assessed by the content in the blood of AST (left panel), ALT (middle panel), and ALP (right panel). The activities of each of these enzymes were elevated within 3 hours. By 6 hours, the activities of AST and ALT had increased 100-fold, whereas that of ALP had doubled. Histologic examination of the livers after 6 hours confirmed the presence of extensive centrilobular necrosis (Figure 2).

Prevention by LSOD of the Liver Necrosis Induced by Acetaminophen

Figure 3 details the dose-dependent prevention by LSOD of the liver necrosis produced within 3 hours by 500 mg/kg body weight of acetaminophen. Doses of LSOD of 64,000 U/kg of body weight or greater given immediately on treating the animals with APAP reduced the activity of AST (left panel), ALT (middle panel), and ALP (right panel) in the blood to their control levels.

Table 1 details that this protection was still evident after 6 hours. In addition, Table 1 gives the results of a number of controls that documented the specificity of the protection by LSOD, which prevented the rise in both AST and ALT produced at 6 hours by 500 mg/kg body weight

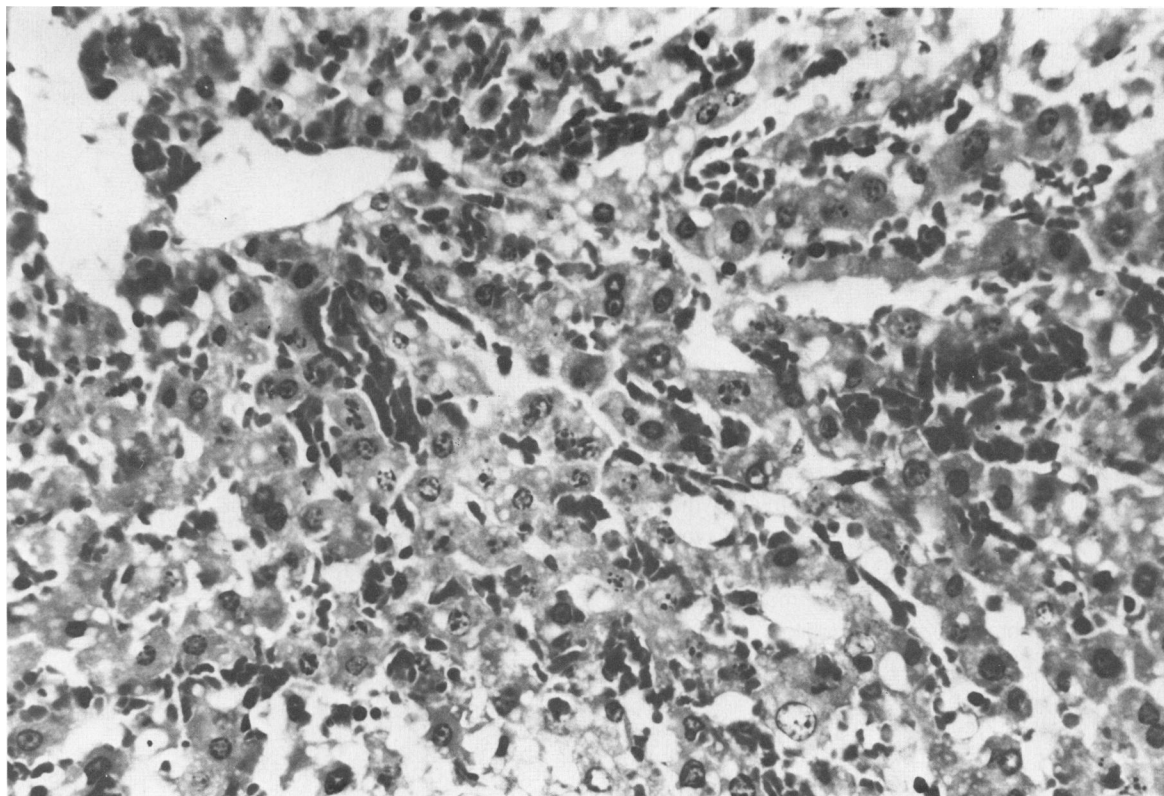


Figure 2. Liver necrosis produced by 500 mg/kg body weight acetaminophen. A photomicrograph of the liver 6 hours after administration of acetaminophen reveals extensive centrilobular necrosis with many hepatocytes exhibiting karyolysis.

of acetaminophen. By contrast, liposomes without encapsulated SOD, free SOD (FSOD), or liposomes encapsulated with heat-denatured SOD (LSODden) had no effect on the extent of liver necrosis induced by acetaminophen. Finally, administration of liposomes, FSOD, LSOD, or LSODden to control rats (no acetaminophen) did not increase AST or ALT in the blood (Table 1).

The protective effect of LSOD was confirmed by histologic examination of the livers. Comparison of Figure 4 with Figure 2 documents the complete absence of the manifestations of liver cell necrosis in the rats that received 500 mg/kg body weight of acetaminophen and LSOD.

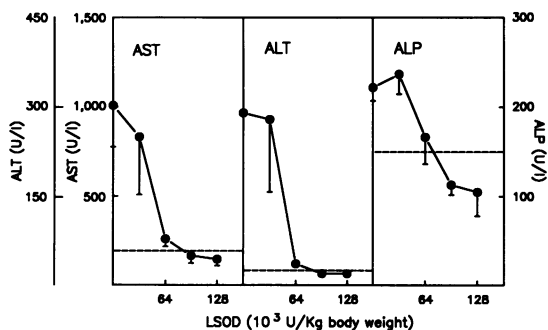


Figure 3. Dose dependency of the prevention by LSOD of the liver cell necrosis produced by 500 mg/kg body weight of acetaminophen. The extent of liver necrosis was determined 3 hours after administering acetaminophen and the indicated doses of LSOD. The dashed line represents the control value. The results are the mean \pm SD of the determinations on five animals. The extent of liver necrosis as assessed by each of the three enzymes was significantly different in the absence of LSOD ($P < 0.02$) with 64,000 U/kg of body weight of LSOD.

Table 1. Prevention by LSOD of the Hepatotoxicity of Acetaminophen (APAP)

Treatment	Liver necrosis		
	AST	(U/l)	ALT
Control	178 \pm 43		27 \pm 4
APAP alone	7,045 \pm 1800		1,199 \pm 313
APAP + LSOD	167 \pm 40		26 \pm 5
APAP + liposomes (no SOD)	6,595 \pm 1765		1,322 \pm 680
APAP + FSOD (no liposomes)	6,051 \pm 919		1,035 \pm 186
APAP + LSODden	6,880 \pm 897		1,084 \pm 320
Liposomes (no APAP)	194 \pm 19		21 \pm 2
LSOD (no APAP)	210 \pm 45		26 \pm 3
LSODden (no APAP)	194 \pm 19		30 \pm 6
FSOD (no APAP)	234 \pm 52		24 \pm 5

APAP was 500 mg/kg body weight; LSOD and FSOD, 96,000 U/kg body weight; LSODden, 24 mg protein/kg body weight; liposomes alone, 168 mg lipid/kg body weight. Extent of liver necrosis was determined 6 hours after treatment with APAP. All results are the mean \pm SD of the determinations on five animals.

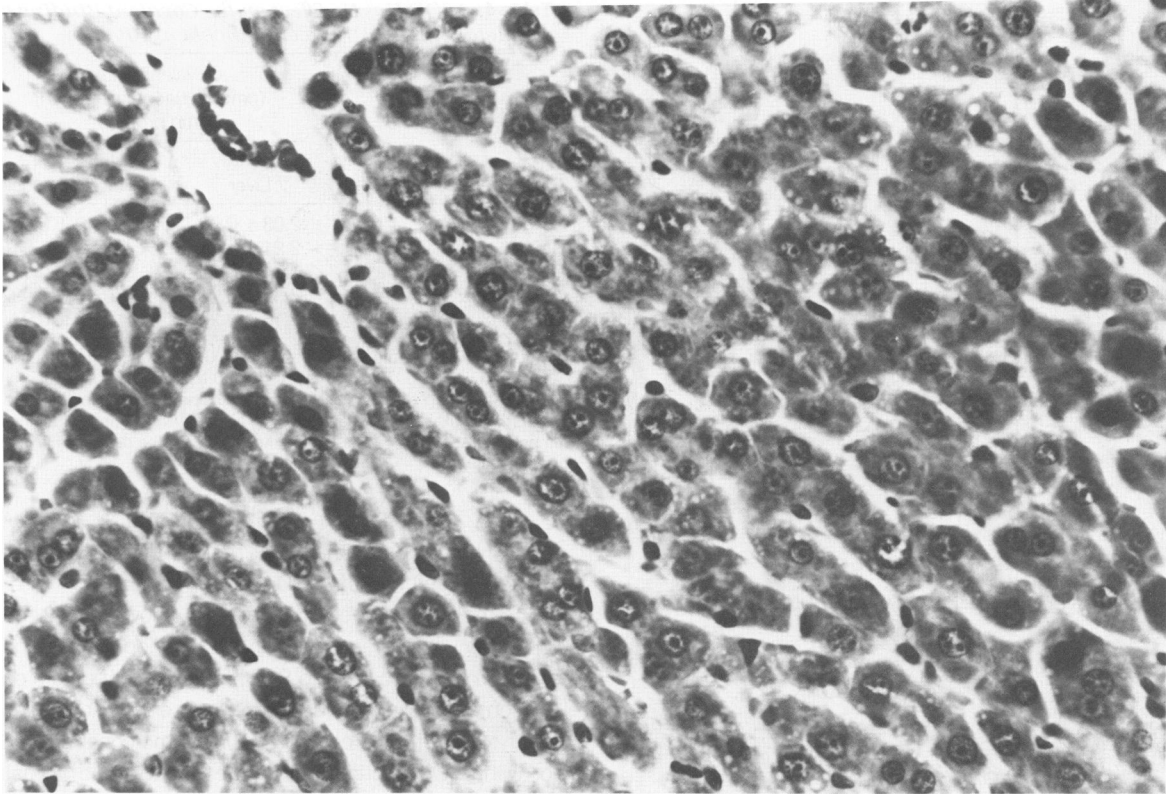


Figure 4. Prevention by LSOD of acetaminophen-induced liver cell necrosis. A photomicrograph 6 hours after the administration of 500 mg/kg body weight of acetaminophen and 96,000 U/kg body weight of superoxide dismutase as LSOD reveals a normal liver.

Importantly, LSOD did not simply delay the development of liver cell necrosis in rats treated with 500 mg/kg body weight of acetaminophen. Rats given a second injection of LSOD at 12 hours were without liver necrosis 24 hours after treatment with 500 mg/kg body weight of acetaminophen (Table 2). Such protection at 24 hours depended on the dose of LSOD (Table 2) and was confirmed by histologic examination of the livers (data not shown). In the absence of the second dose of LSOD, liver necrosis was now evident at 24 hours (Table 2). Finally, the data in Table 2 indicate that neither empty liposomes (no SOD), FSOD, nor LSODden given at both 'zero' time and at 12 hours had a protective effect on the liver necrosis produced by 500 mg/kg body weight of acetaminophen after 24 hours.

LSOD Does Not Prevent the Depletion of Hepatic GSH

The hepatotoxicity of acetaminophen is dependent on its metabolism by the liver. Thus, an inhibition of the metabolism of acetaminophen by LSOD could be the basis of its prevention of the liver necrosis. The effect of LSOD on the

metabolism of acetaminophen was examined in two ways: by the rate of depletion of GSH and by the extent of the covalent binding of the metabolites of [^3H]acetaminophen.

Table 2. LSOD Does Not Simply Delay the Onset of Acetaminophen (APAP)-induced Liver Cell Necrosis

Treatment	Liver necrosis
	AST (U/l)
Control	183 \pm 14
APAP (24 hours)	15,525 \pm 603
APAP + LSOD (96,000 U/kg BW) at 0 and 12 hours	190 \pm 11
APAP + LSOD (64,000 U/kg BW) at 0 and 12 hours	398 \pm 26
APAP + LSOD (32,000 U/kg BW) at 0 and 12 hours	12,645 \pm 988
APAP + LSOD (96,000 U/kg BW) at 0 hour only	15,590 \pm 537
APAP + FSOD at 0 and 12 hours	15,569 \pm 617
APAP + liposomes (no SOD) 0 and 12 hours	15,550 \pm 449
APAP + LSODden at 0 and 12 hours	15,531 \pm 609

APAP was 500 mg/kg of body weight (BW); FSOD, 96,000 U/kg (BW); LSODden, 24 mg denatured enzyme protein/kg (BW); liposomes alone, 168 mg lipid/kg (BW). Extent of liver necrosis was determined 24 hours after treatment with APAP. All results are the mean \pm SD of the determinations on five animals.

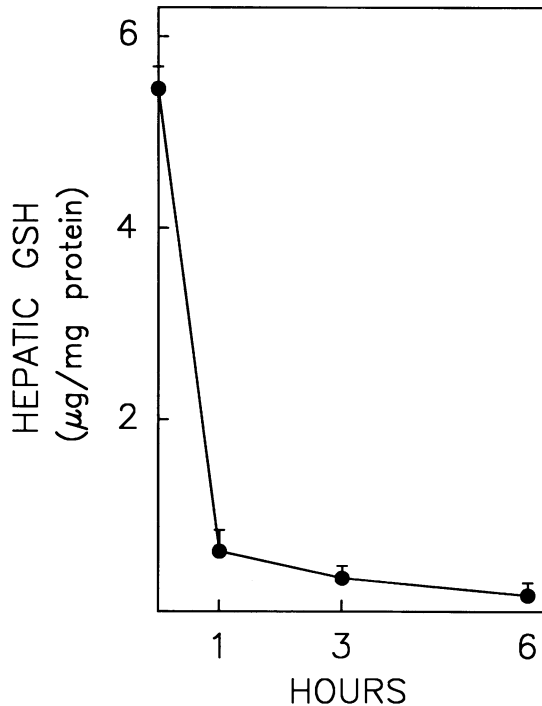


Figure 5. Time course of the depletion of GSH induced by 500 mg/kg body weight of acetaminophen. The results are the mean \pm SD of the determinations on five animals.

Figure 5 illustrates the time course of the depletion of hepatic stores of GSH produced by 500 mg/kg body weight of acetaminophen. Within the first hour, GSH fell to 10% of the initial level and remained at this level throughout the entire 6-hour time course of the experiment. Table 3 documents that LSOD had no effect on this depletion of GSH induced by acetaminophen. After 3 hours there was no difference in the extent of depletion

Table 3. LSOD Does Not Affect the Depletion of GSH Induced by Acetaminophen (APAP)

Treatment	Hepatic GSH ($\mu\text{g}/\text{mg}$ of protein)
Control	5.7 \pm 0.7
APAP	1.3 \pm 0.4*
APAP plus LSOD	1.1 \pm 0.4†
APAP plus liposomes (no SOD)	1.2 \pm 0.4†
APAP plus FSOD	1.1 \pm 0.5†
APAP plus LSODden	1.2 \pm 0.4†
LSOD (no APAP)	5.4 \pm 0.44‡
Liposomes alone (no APAP)	5.4 \pm 0.6‡
FSOD (no APAP)	5.7 \pm 0.9‡
LSODden (no APAP)	5.6 \pm 0.5‡

APAP was 500 mg/kg body weight; LSOD and FSOD, 96,000 U/kg body weight; LSODden, 24 mg denatured enzyme protein/kg body weight; liposomes alone, 168 mg lipid/kg body weight. The GSH content of the livers was determined 3 hours after treatment with APAP. All results are the mean \pm SD of the determinations on five animals.

* Significantly different from the control at $P < 0.001$.
 † $P > 0.3$ compared to APAP alone.
 ‡ $P > 0.3$ compared to control.

Table 4. LSOD Has No Effect on the Covalent Binding of the Metabolites of [^3H]Acetaminophen (APAP)

Treatment	Covalent binding (nmol [^3H]acetaminophen/mg protein)	
	Liver	Medial femoral muscle
APAP	2.03 \pm 0.30	0.49 \pm 0.07
APAP plus LSOD	2.21 \pm 0.31	0.24 \pm 0.04
APAP plus liposome	2.24 \pm 0.28	0.35 \pm 0.16
APAP plus FSOD	2.31 \pm 0.09	0.37 \pm 0.09
APAP plus LSODden	2.18 \pm 0.26	0.22 \pm 0.04

APAP was 500 mg/kg body weight (500 $\mu\text{Ci}/\text{mmol}$ [^3H] (G)); LSOD and FSOD, 96,000 U/kg body weight; LSODden, 24 mg denatured enzyme protein/kg body weight; liposomes alone, 168 mg lipid/kg body weight. The extent of covalent binding was determined 3 hours after treatment with APAP. All results are the mean \pm SD of the determinations on four animals.

of GSH in those animals that received acetaminophen alone or together with LSOD.

Some additional controls are also shown in Table 3. Treatment of the animals with acetaminophen together with empty liposomes (no SOD) had no effect on the depletion of GSH. This was also the case with acetaminophen together with free SOD (not liposome-encapsulated) or with LSODden. Importantly, treatment of the animals with either LSOD, empty liposomes, FSOD, or LSODden in the absence of acetaminophen had no effect on the content of GSH.

LSOD Has No Effect on the Covalent Binding of the Metabolites of Acetaminophen

Table 4 shows the effect of LSOD on the extent of the covalent binding of the metabolites of 500 mg/kg body weight of [^3H]acetaminophen to the proteins of liver and an extrahepatic tissue, skeletal muscle. Several features of the data are noteworthy. Neither LSOD, empty liposomes, FSOD, nor LSODden had any effect on the extent of the covalent binding of the metabolites of [^3H]acetaminophen to total liver cell protein. In addition, the covalent binding to skeletal muscle was substantially lower than that to hepatic protein in every case.

LSOD Prevents the Peroxidation of Lipids Induced by Acetaminophen

The killing of cultured hepatocytes by acetaminophen is accompanied by the peroxidation of lipids.¹⁴ Protection of the cells with the antioxidant *N,N*-diphenyl-*p*-phenylene-diamine had no effect on the metabolism of acetaminophen at the same time that it abolished the lipid peroxidation.¹⁴ These data were interpreted as demonstrating a

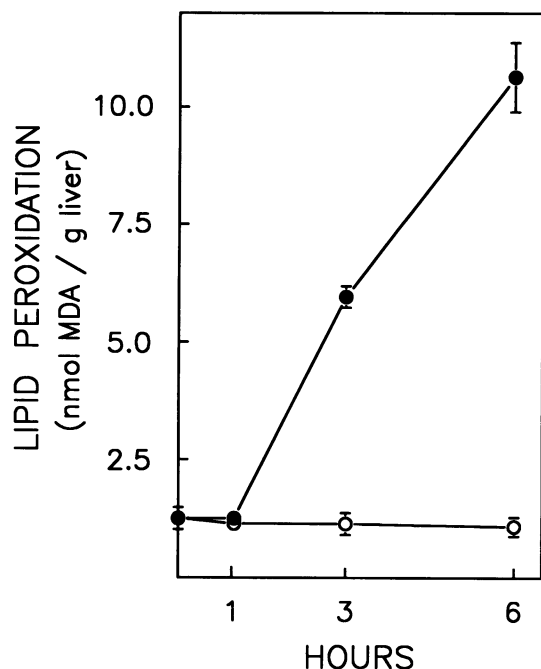


Figure 6. Prevention by LSOD of the peroxidation of lipids induced by 500 mg/kg body weight of acetaminophen. Animals treated with acetaminophen alone (closed circles) or acetaminophen plus LSOD (open circles) were given 96,000 U/kg body weight of superoxide dismutase. The results are the mean \pm SD of the determinations on three or four animals.

role for lipid peroxidation in the hepatotoxicity of acetaminophen.¹⁴ We show here that LSOD prevented the peroxidation of lipids that occurs in the intact rat during the course of the intoxication with acetaminophen.

Figure 6 illustrates the effect of LSOD on the time course the peroxidation of lipids in rats that were intoxicated with 500 mg/kg body weight of acetaminophen. Lipid peroxidation was quantitated in the accumulation of malondialdehyde, which was first detected between 1 and 3 hours after treating the rats with acetaminophen. Within 6 hours, the accumulation of MDA was more than 10 nmol per gram of liver. Liposome-encapsulated human recombinant superoxide dismutase prevented the accumulation of malondialdehyde over the entire 6-hour time course of the experiment illustrated in Figure 6.

Table 5 confirms the specificity of the prevention of lipid peroxidation by LSOD. Empty liposomes, FSOD, or LSODden did not prevent the peroxidation of lipids, a result in agreement with their inability to prevent the liver cell necrosis induced by acetaminophen. In addition, these same reagents had no effect in the absence of acetaminophen on the basal level of MDA.

LSOD Prevents the Potentiation by BCNU of Acetaminophen-induced Liver Injury

Inhibition of glutathione reductase by BCNU potentiates the toxicity of acetaminophen both in the intact rat^{13,20} and

Table 5. Specificity of the Antioxidant Action of LSOD (APAP)

Treatment	Lipid peroxidation (nmol MDA/g liver)
Control	1.3 \pm 0.2
APAP	10.7 \pm 0.7
APAP plus LSOD	1.1 \pm 0.1
APAP plus liposomes (no SOD)	10.0 \pm 0.1
APAP plus FSOD	10.9 \pm 0.8
APAP plus LSODden	10.8 \pm 0.4
LSOD (no APAP)	1.1 \pm 0.1
Liposomes (no APAP)	1.0 \pm 0.1
FSOD (no APAP)	1.0 \pm 0.0
LSODden (no APAP)	1.2 \pm 0.1

Acetaminophen was 500 mg/kg body weight; LSOD and FSOD, 96,000 U/kg body weight; LSODden, 24 mg denatured enzyme protein/kg body weight; liposomes alone, 168 mg lipid/kg body weight. The accumulation of MDA in the livers was determined 6 hours after treatment with APAP. All results are the mean \pm SD of the determinations on three animals.

in primary cultures of hepatocytes.¹¹⁻¹⁴ Table 6 shows that LSOD also prevented the liver necrosis induced by acetaminophen after treatment of the rats with BCNU. In this experiment, the rats were injected with only 200 mg/kg body weight of acetaminophen, to reduce the extent of liver necrosis in the absence of BCNU.

Discussion

The data in the present report document that superoxide anions participate in the liver necrosis produced by acetaminophen. In other words, the data document that the toxicity of acetaminophen in the intact animal is related to an oxidative stress that is imposed by some consequence of the metabolism of this chemical. In addition, the data reproduce in the intact rat an important feature of the killing of cultured hepatocytes by acetaminophen, namely, the ability of SOD to prevent the toxicity of acetaminophen.¹² Thus the current report reinforces our previ-

Table 6. LSOD Prevents the Potentiation by BCNU of the Toxicity of Acetaminophen

Treatment	Liver necrosis		
	AST	(U/l)	ALT
Control	120 \pm 15		21 \pm 4
APAP	209 \pm 1*		38 \pm 2*
BCNU alone	327 \pm 141		59 \pm 29
APAP + BCNU	1,173 \pm 43†		156 \pm 6†
APAP + BCNU + LSOD	467 \pm 25‡		60 \pm 1‡

APAP was 200 mg/kg body weight; LSOD and FSOD, 96,000 U/kg body weight; BCNU 60 mg/kg body weight. The extent of liver necrosis was determined 3 hours after treatment with APAP. All results are the mean \pm SD of the determinations on three or four animals.

* Significantly different from control at $P < 0.01$.

† Significantly different from BCNU alone or from APAP alone at $P < 0.01$.

‡ Significantly different from APAP + BCNU at $P < 0.001$.

ous conclusion²⁰ that the essential features of the hepatotoxicity of acetaminophen in the intact rat are similar to those determining the killing of cultured hepatocytes by the same toxin.

The protection afforded by LSOD is fully attributable to the action of SOD. Increasing protection was obtained with increasing doses of LSOD (Figure 3). Furthermore, empty liposomes or liposomes containing heat-inactivated SOD were ineffective (Table 1). Protection by SOD depended on the encapsulation of the superoxide dismutase. Free SOD did not protect, at least at doses that were fully protective when encapsulated within liposomes (Table 1). Interestingly, free SOD prevents the cell killing of cultured hepatocytes by acetaminophen.¹² This difference between the action of SOD in the intact animal (Table 1) and in cell culture¹² is most likely a dose-dependent phenomenon. Superoxide dismutase protects hepatocytes from the toxicity of acetaminophen at much lower doses when it is encapsulated in a liposome rather than free in the culture medium.²⁶ Similarly, it has been reported that liposome-encapsulated SOD penetrates cells at a much faster rate than does free SOD.²⁷

Prevention by LSOD of the liver necrosis produced by acetaminophen was achieved without inhibition of the metabolism of this toxin. Thus, LSOD did not prevent the acetaminophen-induced depletion of GSH (Table 3) or the covalent binding of the metabolites of [³H]acetaminophen (Table 4). The latter result does not necessarily preclude a role for covalent binding in the hepatotoxicity of acetaminophen. However, it does argue that covalent binding is not sufficient to cause liver necrosis. Furthermore, the conditions of the present study do not necessarily require covalent binding as a step in the genesis of liver necrosis. Covalent binding is most simply explained as an epiphenomenon of the metabolism of acetaminophen.

The data in the present report confirm the previous demonstration that the toxicity of acetaminophen, both in the intact animal⁸⁻¹⁰ and in cell culture,¹⁴ is accompanied by the peroxidation of lipids. Importantly, LSOD prevented this lipid peroxidation in parallel with the prevention of the necrosis. Thus the lipid peroxidation must be a consequence of a mechanism that, on the one hand, superoxide dismutase inhibits and that, on the other, is related to the lethal cell injury. The data presented here do not necessarily indicate that the lipid peroxidation is a part of the same mechanism that kills the cells. However, previous data suggested that lipid peroxidation is causally related to killing of cultured hepatocytes by acetaminophen.¹⁴ It then follows that the lipid peroxidation may similarly be related to the genesis of acetaminophen-induced liver cell necrosis in the intact animal.

In summary, the demonstration that liposome-encapsulated superoxide dismutase prevented the induction of liver necrosis in the intact animal documents the participa-

tion of superoxide anions in the hepatotoxicity of acetaminophen.

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