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Sinusoidal Endothelial Cells as a Target for Acetaminophen Toxicity

DIRECT ACTION VERSUS REQUIREMENT FOR HEPATOCYTE ACTIVATION IN DIFFERENT MOUSE STRAINS

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ABSTRACT. Hepatic congestion occurs early in acetaminophen poisoning. This study examines whether acetaminophen is toxic to sinusoidal endothelial cells (SEC), which might lead to microcirculatory disruption. Acetaminophen toxicity was examined *in vivo* and *in vitro* in SEC and hepatocytes from C3H-HEN and Swiss Webster mice. In both strains, there was significantly more toxicity to SEC than to hepatocytes; in SEC from C3H-HEN mice, acetaminophen was directly toxic, but the presence of hepatocytes was required for toxicity to Swiss SEC. Acetaminophen, 750 mg/kg, by gavage caused toxicity with variability within and between strains, but all animals died between 3.5 and 6 hr with zone 3 hemorrhagic necrosis. Pretreatment of C3H-HEN SEC with aminobenzotriazole, a suicide inhibitor of P450, abolished toxicity. Baseline glutathione (GSH) levels were comparable, but a 12-hr incubation with acetaminophen decreased GSH by 60 and 8%, respectively, in C3H-HEN and Swiss SEC in single cell type culture. In co-culture, under conditions where Swiss SEC viability declined by 73%, hepatocyte viability and GSH only decreased by 21 and 20%, respectively. In conclusion, acetaminophen was toxic to SEC. It was directly toxic to SEC in one mouse strain and required hepatocyte activation in another strain. The lack of direct toxicity to Swiss SEC may be due to the lack of an activating P450 isozyme. Zone 3 hemorrhagic necrosis *in vivo* was comparable in both strains, despite differences in the pathways leading to SEC toxicity *in vitro*. We propose that toxicity to SEC may contribute to hepatic congestion in acetaminophen intoxication. BIOCHEM PHARMACOL53;9:1339–1345, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. acetaminophen; endothelium; liver; cells-cultured; mice

Hepatic congestion is a common feature in acetaminophen intoxication, both in humans [1–6] and in experimental animals [7–12]. The mouse is particularly useful for studying this phenomenon since up to half of the red blood cells of the animal can accumulate in the liver in acetaminophen poisoning [11]. Since the circulatory disruption precedes hepatocyte necrosis, Walker *et al.* [12, 13] have postulated that acetaminophen-induced hepatic congestion contributes to the parenchymal dysfunction in addition to the direct toxic effect to the hepatocyte. In the murine model of acetaminophen toxicity. *N*-Acetylcysteine prevents the disturbance of the hepatic circulation and sustains hepatic function, which has been considered further support for a role of microcirculatory disruption in the injury [11, 12, 14].

One explanation for the microcirculatory disturbance has been neutrophil plugging [15]. However, there is evidence to suggest that neutrophil accumulation is not a prominent feature of acetaminophen intoxication [16]. An alternate explanation is that in addition to the toxicity to the hepatocyte, acetaminophen is also toxic to the SEC§, either due to the action of toxic metabolites released from hepatocytes or generated directly in the SEC. Among the earliest findings by scanning electron microscopy after acetaminophen poisoning is the appearance of large pores in the SEC and enlargement of the space of Disse with accumulation of erythrocytes [13]. SEC separate partially from, but remain attached to, underlying hepatocytes. Eventually the sinusoidal lumen collapses, possibly due to compression by the enlarged space of Disse. The occurrence of damage to SEC is supported by the clinical observation that increased serum hyaluronate levels are seen in patients with acute liver damage due to acetaminophen [17]. Hyaluronic acid is

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§ *Abbreviations:* MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's minimal essential medium; LSD, least significant difference; NAPQI, *N*-acetyl-*p*-benzoquinone imine; SEC, sinusoidal endothelial cell(s); and GSH, glutathione.

cleared from the circulation by SEC, and decreased hyaluronate clearance has been used as a marker for sinusoidal endothelial cell damage.

To determine whether acetaminophen might disrupt the hepatic microcirculation through toxicity to SEC, the present study examines metabolism and toxicity of acetaminophen in SEC, hepatocytes, and both cell types in co-culture.

MATERIALS AND METHODS

All chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Animals

Two strains of mice were used: C3H-HEN, an inbred species, and Swiss Webster, an outbred species. Eight-week-old C3H-HEN male mice were obtained from Bantin and Kingman Laboratories (Fremont, CA), and male Swiss Webster mice (20–28 g) were obtained from Simonsen (Gilroy, CA). All protocols dealing with animals were reviewed and approved by the Animal Care and Use Committe at the University of Southern California to ensure ethical and humane treatment of the animals. This study followed the guidelines outlined in the NIH "Guide for the Care and Use of Laboratory Animals" (Revised 1985) prepared by the National Academy of Sciences.

Cell Culture

Endothelial cells and hepatocytes were isolated as previously described [18]. Viability by trypan blue exclusion was usually greater than 95 and 90%, respectively. SEC were seeded in DMEM-low glucose (Irvine, Santa Ana, CA) and hepatocytes were seeded in DMEM/F12 (Irvine). Plating medium was supplemented with 10% fetal bovine serum. All culture medium was supplemented with 14.6 mM HEPES buffer, 2 mM L-glutamine, and penicillin-streptomycin solution (25,000 U penicillin and 25 mg streptomycin/500 mL medium).

Drug Studies

Co-culture experiments were done using special inserts in the wells. One cell type was plated in the well and the other cell type was plated in the porous insert. Thus, the two cell types shared the same medium but without mixing of cells, and viability of each cell type could be examined separately. Both cell types were plated and allowed to adhere for 2 hr prior to drug incubation. SEC were seeded on collagen-coated inserts for 24-well plates (Transwell-Col, Costar, Cambridge, MA) at a density of 250,000 cells/insert. These cells were exposed to acetaminophen in DMEM-F12 with 10% fetal bovine serum for 17 hr in the presence (co-culture) or absence of hepatocytes seeded in the wells of 24-well plates coated with rat tail collagen at a density of 200,000 cells/well. At the end of the incubation, viability was tested using the MTT assay [19, 20]. This assay was performed as previously described and has been validated in murine hepatocytes and sinusoidal endothelial cells [18]. For viability testing of cells in co-culture, the two cell types were separated by placing the inserts in a separate multiwell dish before the incubation with MTT. For co-culture studies in which hepatocyte GSH was measured, studies were done in 6-well co-culture plates (Costar).

Acetaminophen toxicity studies with aminobenzotriazole and supplemental GSH were done in 96-well plates with SEC seeded at a density of 400,000 cells/cm^2 and hepatocytes seeded at a density of 125,000 cells/cm^2 in 96-well plates on rat tail collagen.

To rule out collateral toxicity in the co-culture experiments, hepatocytes were killed by air-drying. After cell attachment, medium was aspirated and hepatocytes were incubated in the tissue culture hood for 15 min, followed by a 30-min incubation in the incubator. Hepatocytes and SEC were then co-cultured for 17 hr.

In Vivo *Studies*

Mice were given acetaminophen (750 mg/kg) or a saline vehicle by gavage (0.5 to 0.7 mL vol.). Mice were killed under pentobarbital anesthesia by cervical dislocation. Sections were stained with hematoxylin and eosin and with Gomori's trichrome. Stained tissue sections were coded and reviewed without knowledge of the treatment or mouse strain by one of us (H.M.S.).

GSH Assays

GSH levels were measured in cells seeded in 6-well multiwell plates at a density of 400,000 cells/cm^2. GSH was measured in SEC by the high performance liquid chromatographic method utilizing fluorimetric detection [21–24] with the previously described modifications [25]. Hepatocyte GSH was measured by the method of Tietze [26]. GSH was normalized to the amount of cellular protein present. Cellular protein was measured in a 96-well microplate on a Bio-Rad microplate reader as previously described [18].

Statistics

All data are expressed as means ± SEM from at least three separate experiments employing cells from different isolations. All measurements were done in triplicate wells. Raw data are displayed in Fig. 1. For the sake of clarity the subsequent figures are displayed as percentages of control values, which were stable for both cell types throughout the experiments. Statistical analysis was done using the Microsoft Excel Analysis ToolPak. Concentration–response curves were compared with two-factor repeated measures ANOVA. When concentration–response curves were statistically significant by ANOVA, *a posteriori* comparison of individual doses between concentration–response curves

was done by LSD [27]. Some comparisons were done by either paired or an unpaired *t*-test as indicated. A *P* value of less than 0.05 was considered significant.

RESULTS

In studies with cells isolated from Swiss Webster mice (Fig. 1A), acetaminophen (10 mM) was not directly toxic to SEC alone, but was toxic in co-culture. In contrast, acetaminophen was directly toxic to SEC isolated from C3H-HEN mice (Fig. 1B), and toxicity was not enhanced by co-culture with hepatocytes. There was significantly more toxicity to SEC than to hepatocytes in both strains of mice, although this required the presence of hepatocytes in Swiss mice. Acetaminophen was significantly more toxic to hepatocytes from C3H-HEN mice than from Swiss mice ($P = 0.02$ by *t*-test).

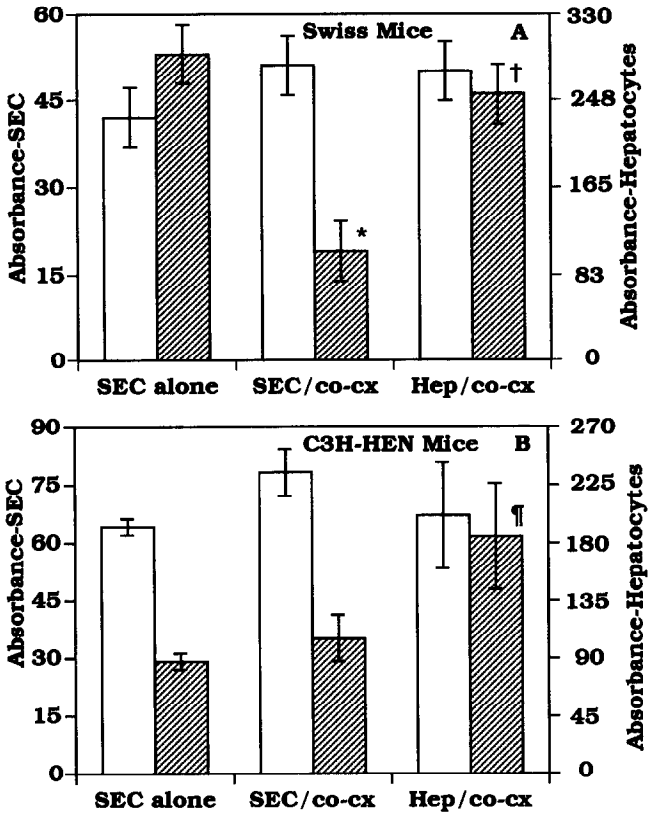

FIG. 1. Acetaminophen toxicity in SEC and hepatocytes from two strains of mice. The toxicity of 10 mM acetaminophen was examined in SEC cultured alone, and SEC and hepatocytes in co-culture. Cells were exposed to medium (open bars) or 10 mM acetaminophen (hatched bar). Left ordinate represents absorbance for SEC, right ordinate absorbance for hepatocytes. Panel A: Cells isolated from Swiss Webster mice. ANOVA: $P < 0.01$ SEC alone vs co-culture, *$P < 0.005$ by LSD, N = 3; ANOVA: $P < 0.0001$ hepatocytes vs SEC in co-culture, †$P < 0.001$ by LSD, N = 5. Panel B: Cells isolated from C3H-HEN mice. ANOVA, N = 6: P = NS SEC alone vs SEC in co-culture; $P \leq 0.0001$ for hepatocytes vs SEC in co-culture or alone, ¶$P < 0.005$ by LSD. Values are means ± SEM.

Co-cultures of SEC and hepatocytes were also done across the strains. SEC from C3H-HEN mice sustained the same degree of toxicity in the presence or absence of hepatocytes from either mouse strain, whereas in SEC from Swiss mice a similar degree of toxicity was induced in the presence of hepatocytes from either Swiss or C3H-HEN mice (data not shown). This demonstrates that the susceptibility is intrinsic to the SEC, not due to differences in metabolism by hepatocytes from different strains.

One possible confounder in co-culture experiments might be that dead hepatocytes could kill SEC in co-culture through a non-specific mechanism. To rule this out, hepatocytes were killed by air-drying and then placed in co-culture with SEC for 17 hr. Viability of SEC in co-culture with control hepatocytes was the same as that of SEC in co-culture with hepatocytes killed by air-drying.

In vivo studies were done to determine whether there was any difference in the response between C3H-HEN and Swiss Webster mice to acetaminophen (750 mg/kg) given by gavage (Table 1). Acetaminophen-treated animals from both strains were killed at 1, 2, 3, and 4 hr. Five additional mice were followed until spontaneous death ensued. Body weight of the mice before treatment and at the end of the experiment was unchanged (data not shown) at all time points. However, liver weight increased approximately 2-fold at 4 hr (liver weight in control littermates killed between 1 and 4 hr after saline gavage: C3H-HEN mice: 1.78 ± 0.10 g; Swiss mice: 1.28 ± 0.07 g, N = 6). The livers of animals killed in the first 3 hr all appeared normal in contrast to the livers of all four animals in the 4-hr group and those followed until spontaneous death, which were dark red and engorged. Histological details are summarized in Table 1. At 1 hr, livers from acetaminophen-treated mice showed no change. After 2 and 3 hr the progression of injury varied in individual animals of either strain. In the C3H-HEN mice, onset of hemorrhagic necrosis occurred at 3 hr with varying extent of damage in the two mice killed. Hemorrhagic necrosis was characterized by spilling of blood from the distorted sinusoids into what was formerly the hepatocyte cords. In one Swiss mouse, the sections demonstrated a transitional stage between normal and hemorrhagic necrosis at 2 hr, with shrunken and vacuolated hepatocytes and a thin rim of hemorrhage between the normal hepatocytes in zone 1 and the damaged hepatocytes in zone 2. However, sections from the remaining three Swiss mice killed 2–3 hr after treatment only showed zone 3 hepatocyte pallor and vacuolization. By 4 hr, both Swiss mice and one of the two C3H-HEN mice had died, and the remaining mouse was killed. Histological examination of livers from both strains showed zone 3, and sometimes zone 2, hemorrhagic necrosis and fatty change in zone 1. Death occurred between 3.5 and 6 hr in the five mice followed until spontaneous death. All five demonstrated zone 3 hemorrhagic necrosis, which in some cases also spread to zone 2.

SEC were treated with aminobenzotriazole, a suicide inhibitor of a wide range of P450 isozymes [28–31]. Pretreat-

TABLE 1. Comparison of response to acetaminophen in two mouse strains

Strain	Death[a]	Body wt (g)	Liver wt (g)	Histological description
C3H	1 hr	28.5	1.7 (6.0)*	no change; *note:* this is a saline control animal
Swiss	1 hr	29.21	1.1 (3.8)	no change; *note:* this is a saline control animal
C3H	1 hr	31.8	1.8 (5.7)	no change
C3H	1 hr	28.3	1.3 (4.6)	no change
Swiss	1 hr	29.3	2.3 (7.5)	no change
Swiss	1 hr	31.4	1.7 (5.4)	no change
C3H	2 hr	29.2	1.9 (6.5)	mild z 3† hepatocyte vacuolization
C3H	2 hr	29.5	1.9 (6.4)	mild-to-moderate z 3 vacuolization; focal hepatocytolysis
Swiss	2 hr	30.8	2.2 (7.1)	transition between normal and hemorrhagic necrosis: z 3 hepatocytes with shrunken, vacuolated cytoplasm, rim of hemorrhage at z ½ interface
Swiss	2 hr	29.8	2.0 (6.7)	z 3 hepatocyte pallor, mild vacuolization
C3H	3 hr	30.6	1.7 (5.5)	z 3 hepatocytolysis, congestion, loss of sinusoidal cells
C3H	3 hr	31.2	2.4 (7.7)	z 3 hemorrhagic necrosis
Swiss	3 hr	36.5	2.0 (5.5)	z 3 pallor and vacuolization
Swiss	3 hr	36.9	2.2 (6.0)	crumbling z 3 hepatocytes, loss of sinusoidal cells
C3H	3.75 hr	32.1	3.2 (10.0)	z 3 necrosis, hemorrhage z ⅔ interface; spontaneous death
C3H	4 hr	29.9	3.1 (10.3)	z 3 hemorrhagic necrosis
Swiss	3.5 hr	29.8	3.4 (11.4)	z 3 hemorrhagic necrosis; spontaneous death
Swiss	4.0 hr	30.1	3.2 (10.6)	z 3 hemorrhagic necrosis; spontaneous death
C3H	4.2 hr	27.9	2.37 (8.5)	z 3 hemorrhagic necrosis sparing layer of perivenular hepatocytes
C3H	5.0 hr	27.6	2.3 (8.3)	z ⅔ hemorrhagic necrosis, fatty change z 1
C3H	6.0 hr	28.2	2.37 (8.4)	z ⅔ hemorrhagic necrosis, fatty change z 1
Swiss	3.5 hr	21.5	1.8 (8.4)	z ⅔ hemorrhagic necrosis, fatty change z 1
Swiss	4.2 hr	21.8	2.0 (9.2)	z ⅔ hemorrhagic necrosis, fatty change z 1

Mice received acetaminophen (750 mg/kg) by gavage or saline vehicle. The bottom five mice were followed until death, and the remaining mice were killed 1–4 hr after gavage. Three of four animals in the 4-hr time group died spontaneously, at times indicated. Body weight and liver weight were obtained at the end of the experiment.

* Values in parentheses are percentage of body weight.

† Zone 3 (z 3).

ment with aminobenzotriazole for 1 hr followed by 17-hr co-treatment completely abolished all toxicity of acetaminophen in SEC from C3H-HEN mice (Fig. 2).

The ability of acetaminophen to deplete GSH was examined in single cell cultures of SEC from the two strains (Fig. 3). Two control wells and two wells with cells incubated with acetaminophen were examined by trypan blue dye exclusion every hour to determine the onset of cell death. When trypan blue staining first showed increase in cell death compared with control cells, GSH was measured in the parallel plates. Uptake of trypan blue occurred in SEC from C3H-HEN mice after 13 ± 0.3 hr (N = 5). GSH decreased progressively in the time period prior to onset of toxicity in SEC from C3H-HEN mice. At the time trypan blue uptake first increased above control, GSH in C3H-HEN mice was depleted by 63.4 ± 3.3%. Since SEC from Swiss Webster mice did not sustain toxicity from acetaminophen, GSH levels were also measured at 13 hr. In Swiss Webster mice, SEC GSH levels showed only a 7.5 ± 0.8% decline compared with control cells.

In the co-culture experiment with cells from the Swiss Webster strain, 17-hr incubation with acetaminophen induced little toxicity in hepatocytes, but SEC showed substantial loss of viability. Since the toxic metabolite was presumably being formed in the hepatocyte and exported, we examined GSH status in hepatocytes under these conditions (Table 2). In co-culture plates, SEC viability and hepatocyte GSH were measured after incubation with 10 mM acetaminophen. Viability of hepatocytes was measured in a parallel plate. After a 17-hr incubation, SEC viability had declined by 73%, whereas hepatocyte GSH and viability had decreased by 20 and 21%, respectively.

Previous studies have shown that exogenous GSH can prevent the toxicity of certain compounds to SEC [18, 25]. To determine whether exogenous GSH would also protect SEC from acetaminophen, SEC isolated from C3H-HEN mice were incubated for 17 hr with acetaminophen plus 0.5 mM GSH. The addition of 0.5 mM GSH abolished all toxicity from acetaminophen (Fig. 4).

DISCUSSION

Acetaminophen is toxic to SEC from both strains of mice, but by two different mechanisms. In C3H mice, acetaminophen is directly toxic to SEC via P450 activation. Acetaminophen is as toxic to SEC from Swiss Webster mice as it is to SEC from C3H mice, but in SEC from the Swiss Webster mice this requires metabolic activation by hepatocytes. Regardless of the pathway leading to SEC toxicity, both strains developed zone 3 hemorrhagic necrosis in a comparable fashion, albeit with more individual variation in the time–course in the Swiss mice.

The ability of ABT to completely block acetaminophen toxicity in SEC from C3H-HEN suggests that activation is

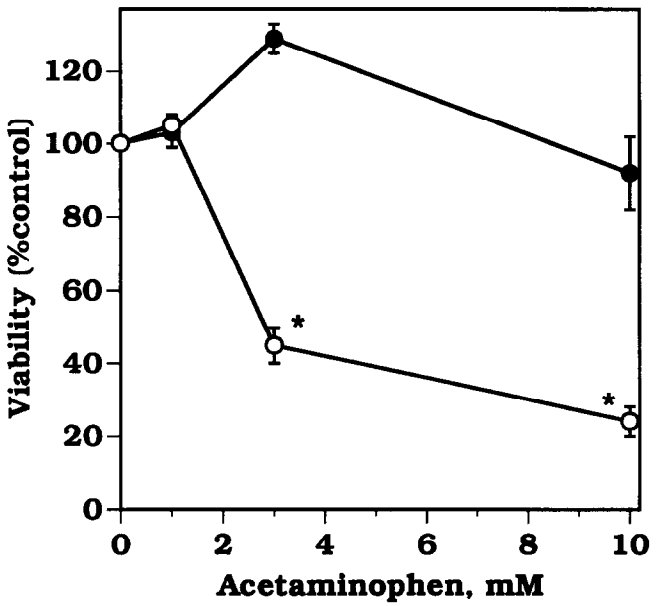

FIG. 2. Effect of aminobenzotriazole on acetaminophen toxicity in SEC from C3H-HEN mice. Comparison of acetaminophen concentration–response curves with (●) versus without (○) aminobenzotriazole by ANOVA was statistically significant ($P < 0.0001$; N = 3). Key: *$P < 0.001$ by LSD at 3 and at 10 mM, with versus without aminobenzotriazole. Values are means ± SEM.

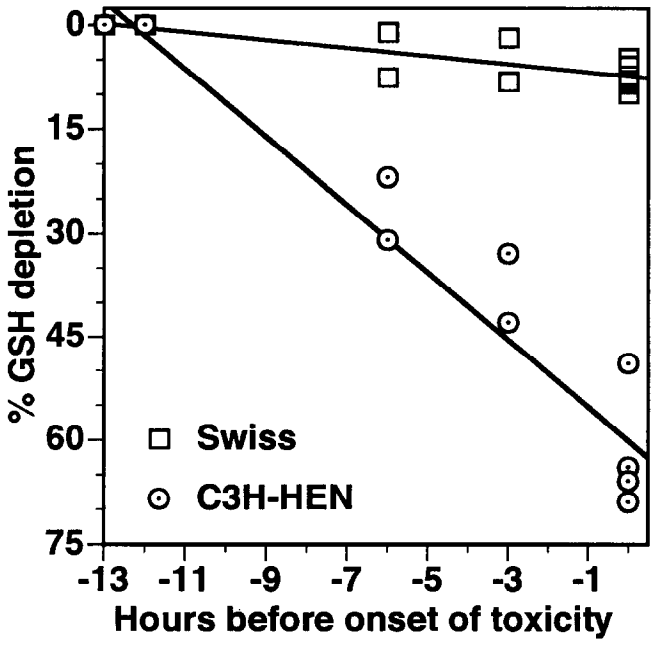

FIG. 3. GSH levels in SEC exposed to acetaminophen. GSH was measured at various time-points before onset of toxicity. Onset of toxicity (t = 0) was determined to be 13 ± 0.3 hr in SEC from C3H-HEN mice by trypan blue dye exclusion (data not shown). Data were obtained from 5 cell preparations; each point represents the mean of duplicate measurements. Comparison between the two mouse strains by ANOVA: $P < 0.0001$. Comparison of the two strains at individual time-points: $P < 0.005$ at -6 hr and $P < 0.001$ at -3 and 0 hr by LSD.

TABLE 2. Acetaminophen effect on hepatocyte GSH and on cell viability

	Hepatocyte GSH (nmol/mg protein)	Viability (%)	
		Hepatocytes	SEC
Control	43.6 ± 6.2	100	100
Treated	35.2 ± 6.3	79.3 ± 2.9*	27.0 ± 1.6*
% Decrease†	20.1 ± 3.2	20.7 ± 2.9	73.0 ± 1.6

SEC and hepatocytes isolated from Swiss Webster mice were exposed to acetaminophen (10 mM) overnight. Hepatocyte GSH and viability of SEC were measured in cells in co-culture, and viability of hepatocytes was determined in a parallel plate (N = 3). Values are means ± SEM.

* Viability is expressed as a percentage of the simultaneous control cells.

† Percent decrease of treated cells compared with control cells.

via P450. This is consistent with previous demonstrations of P450 activity in SEC [32, 33]. The lack of toxicity in SEC from Swiss Webster mice is likely due to a lack of the appropriate P450 isozyme, CYP2E1 and/or CYP1A2. This is further supported by the data on GSH depletion in SEC from the two strains. The formation of NAPQI from acetaminophen leads to significant GSH depletion. Thus if a cell metabolizes acetaminophen, this is accompanied by a decline in GSH. The lack of substantial decline in SEC GSH levels in Swiss Webster mice further supports the contention that this cell type is unable to metabolically activate acetaminophen.

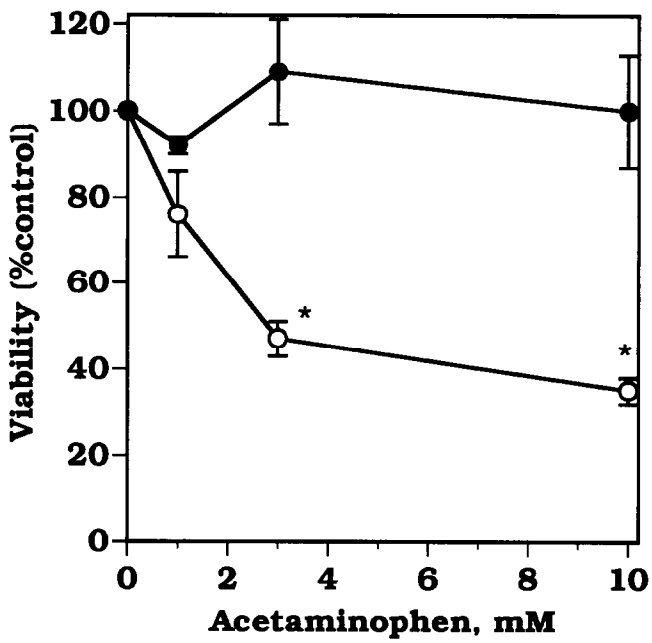

FIG. 4. Effect of addition of exogenous GSH on acetaminophen toxicity in SEC isolated from C3H-HEN mice. Comparison of acetaminophen concentration–response curves with (●) versus without (○) GSH, 0.5 mM, by ANOVA was statistically significant ($P < 0.0001$; N = 3). Key: *$P < 0.001$ by LSD at 3 and at 10 mM, with versus without GSH. Values are means ± SEM.

In the co-culture experiments with Swiss Webster mice, there was significantly more toxicity to SEC than to hepatocytes although the toxicity to SEC was presumably due to NAPQI released from the hepatocytes. We therefore asked the question whether hepatocyte GSH had to be depleted substantially before toxicity occurred in the neighboring SEC. Under conditions that caused severe toxicity to SEC in co-culture, there was only a modest decrease in hepatocyte GSH, and the decrease in GSH paralleled loss of viability. Two possible scenarios might explain these findings. One possibility is that the 20% decline in hepatocyte GSH occurred across the board in all hepatocytes. This would suggest that NAPQI can be exported from the cell before fully depleting cytosolic GSH. The other possibility is that the decrease in GSH may be accounted for by the hepatocytes that died subsequently. In this case, one might envision that there is a subpopulation of metabolically active hepatocytes. In these hepatocytes acetaminophen profoundly depleted GSH, leading to the death of those hepatocytes with release of toxic metabolite that targets the neighboring endothelial cells, most likely before loss of hepatocyte viability.

Exogenous GSH protected SEC from acetaminophen toxicity. The ability of exogenous GSH to protect SEC from toxins was demonstrated previously for dacarbazine, azathioprine, and monocrotaline and may be due to uptake of intact GSH [18, 25]. The hepatocyte is the main source of plasma GSH, which enters the bloodstream across the space of Disse. Thus, GSH concentrations in the space of Disse may be quite substantial. Although it is unlikely that under normal circumstances SEC would take up GSH against the concentration gradient, GSH in the space of Disse may sustain SEC GSH when intracellular levels are depleted. Thus, under conditions of toxin-induced stress, strategies to increase hepatocyte GSH and its efflux may also sustain SEC GSH.

These findings demonstrate that the SEC is a target for acetaminophen *in vitro*. It remains to be determined whether this *in vitro* toxicity is indeed related to the congestion found *in vivo*. Clearly, the toxicity observed *in vivo* occurs much earlier than *in vitro* toxicity to either SEC or hepatocytes. One possible explanation for the temporal difference in *in vitro* toxicity to SEC and *in vivo* injury might be that microcirculatory disruption follows sublethal injury to SEC, which might occur earlier than cell death. Another possibility is that shear stress *in vivo* accelerates or potentiates acetaminophen-induced toxicity. Future studies will need to reconcile *in vitro* findings with *in vivo* events.

Until recently, studies of liver disease have focussed on the hepatocyte as the sole target of toxic insults. However, the contribution of nonparenchymal cells to liver disease is being increasingly recognized. Damage to SEC seems to be a limiting factor in cold preservation of livers for liver transplantation [34–36]. In an experimental animal model of nodular regenerative hyperplasia, abnormalities of SEC precede hepatocyte atrophy and nodular regeneration [37]. Finally, *in vitro* studies suggest that the SEC is more susceptible to toxins that cause hepatic venoocclusive disease than the hepatocyte and that the SEC may therefore be the initial target of these toxins [18, 25, 38]. As we begin to find new diseases in which the SEC may contribute to pathology, there will be an increased impetus to improve our understanding of the unique features of this highly specialized endothelial cell.

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