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EVIDENCE *IN VIVO* FOR ELEVATION OF INTRACELLULAR FREE Ca^{2+} IN THE LIVER AFTER DIQUAT, ACETAMINOPHEN, AND CCl_4*

JANICE O. TSOKOS-KUHN†
Department of Medicine, Center for Experimental Therapeutics, Baylor College of Medicine, Houston, TX 77030, U.S.A.

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Abstract—Several hepatotoxic agents with varied chemical mechanisms of toxicity (acetaminophen, diquat, and CCl_4) depress membrane calcium pumps and/or enhance the permeability of membranes to calcium. To probe the relevance of these findings to maintenance of calcium homeostasis after toxins *in vivo*, we measured the activity of glycogen phosphorylase *a*, as an index of cytosolic free [Ca^{2+}], in freeze-clamped liver samples obtained at several times after the toxin dose. Both acetaminophen and diquat caused significant increases of phosphorylase *a* activity, and activity remained elevated for several hours after the dose. Significantly, the administration prior to diquat of desferrioxamine, which offers protection against the liver necrosis and depression of microsomal Ca^{2+} accumulation observed after diquat alone (Tsokos-Kuhn *et al.*, *Mol Pharmacol* **34**: 209–214, 1988), decreased phosphorylase activation. Activation of phosphorylase was observed also after CCl_4 administration, as previously reported by Long and Moore (*Biochem Pharmacol* **35**: 4131–4137, 1986). We conclude that perturbations in liver membrane Ca^{2+} regulation observed after administration of these hepatotoxins *in vivo* correlate directly with phosphorylase *a* activity, thereby providing additional *in vivo* evidence for an alteration of Ca^{2+} homeostasis early in the development of the liver damage produced by these chemicals.

Considerable attention has been given of late to the hypothesis that alterations in intracellular Ca^{2+} homeostasis may be part of a common pathway of lethal cell injury in toxic liver damage. The alkylating hepatotoxins acetaminophen, bromobenzene, and CCl_4 produce marked decreases in ATP-driven Ca^{2+} accumulation of Sprague–Dawley rat liver plasma membrane vesicles isolated after dose *in vivo* [1]. Hepatotoxic doses of CCl_4 increase the plasma membrane permeability to Ca^{2+} [2] as well. Conversely, diquat, which produces massive oxidative stress and liver necrosis in the male Fischer-344 rat, alters ATP-driven Ca^{2+} accumulation and Ca^{2+} permeability of Fischer rat liver microsomes, whereas male Sprague–Dawley rats exhibit neither liver necrosis nor altered microsomal function after similar treatment [3].

Much of the data that directly support the hypothesis of a key role of altered Ca^{2+} homeostasis derives from studies in isolated cells, however, and there are some apparent differences in the biochemistry of toxic cell death *in vitro* and *in vivo* (e.g. the massive protein thiol oxidation observed during exposure of hepatocytes to redox cycling compounds [4, 5] versus no evidence for oxidation of protein thiols in diquat-induced liver necrosis *in vivo* [6]; and the apparent role of lipid peroxidation in acetaminophen cytotoxicity *in vitro* [7, 8] versus no evidence for lipid peroxidation in acetaminophen hepatic necrosis *in vivo* [9]). Thus, a means of evaluating possible alterations in Ca^{2+} homeostatic control *in vivo* is required. Here we present a study of glycogen phosphorylase activation, as a function of time after dose of acetaminophen, diquat, or CCl_4, to provide a means of detecting changes in cytosolic [Ca^{2+}] *in vivo*. Glycogen phosphorylase is activated by a Ca^{2+}-dependent phosphorylase kinase and has often been used as an indicator of cytosolic free Ca^{2+} levels. Acetaminophen, diquat, and CCl_4 each produce liver injury by a different chemical mechanism, yet all share in common the ability to alter membrane Ca^{2+} handling. Moreover, the data presented here suggest that a rise in intracellular free Ca^{2+} does accompany, and with similar time courses, the hepatotoxic effects of each of these compounds.

METHODS

Animals and toxin treatments. Male Sprague–Dawley rats (175–250 g; Harlan Sprague–Dawley, Inc., Houston, TX) were dosed with acetaminophen (2 g/kg) in 20% Tween 80, p.o. 3-Methylcholanthrene (20 mg/kg) was given i.p. in corn oil 24 hr before acetaminophen. Animals were fasted overnight; some animals were provided 5% glucose water *ad lib.* to maintain liver glucose. CCl_4 (2 ml/kg, 1:1 in corn oil, i.p.) was given to fasted or fed male Sprague–Dawley rats (175–250 g). Fifteen minutes prior to taking the liver samples, animals were injected i.p. with sodium pentobarbital, 50 mg/kg. At the selected time after the dose of hepatotoxin, a midline incision was rapidly made to expose the median liver lobe, and a portion of the tissue was freeze-clamped between liquid nitrogen-cooled aluminium blocks. Fed male Fischer-344 rats (175–250 g; Harlan Sprague–Dawley, Inc., Houston, TX) were dosed with diquat dibromide (0.1 mmol/kg, i.p., in saline), and liver samples were obtained in the same way. In one series of experiments, des-

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† Correspondence: Dr Janice O. Tsokos-Kuhn, Stillmeadow, Inc., 9525 Town Park Drive, Houston, TX 77036.

ferrioxamine (0.24 mmol/kg) was injected i.p. in saline 30 min prior to diquat injection. Liver samples were stored in liquid nitrogen overnight before phosphorylase assays.

Phosphorylase determinations. Phosphorylase *a* activity and total phosphorylase (*a* + *b*) were determined by an assay involving incorporation of α-D-[^{14}C-U]-glucose-1-phosphate into glycogen as described by Tan and Nuttall [10] in weighed portions of freeze-clamped liver samples subjected to Polytron homogenization while still frozen. A given experiment generally included two to three rats at each time point and each assay was run in duplicate. Typically, three to six rat livers were sampled in total per time point (actual N values are shown in the figures). Protein concentrations were determined by the Lowry method, using bovine serum albumin (Type V, Sigma Chemical Co., St Louis, MO) as the protein standard. Phosphorylase activities measured were slightly lower than previously reported, but ratios of phosphorylase *a*: total phosphorylase in fed and fasted animals averaged 60 and 50%, respectively, as reported by Tan and Nuttall [10].

Preparation and permeability assay of plasma and microsomal membranes. Liver plasma membranes were isolated as described [1, 2] 30, 60, or 90 min after administration of 2 ml CCl_4/kg (50% in corn oil, i.p.) to fasted male Sprague–Dawley rats. Ca^{2+} permeability was measured after passive loading of the membrane vesicles as in Ref. 2. Liver microsomes were isolated as described by Moore and coworkers [11] at 30, 60, or 90 min after CCl_4 administration, and Ca^{2+} permeability was measured in the same way as for plasma membranes but after 2 hr rather than 18 hr passive loading.

Chemicals. Diquat dibromide was the gift of Dr Ian Wyatt of Imperial Chemical Industries, Ltd. CCl_4, acetaminophen, and 3-methylcholanthrene were obtained from the Eastman Kodak Co. (Rochester, NY). α-D-[^{14}C-U]Glucose-1-phosphate was purchased from New England Nuclear (Boston, MA). Desferrioxamine was obtained from the college hospital pharmacy. Other chemicals were products of the Sigma Chemical Co.

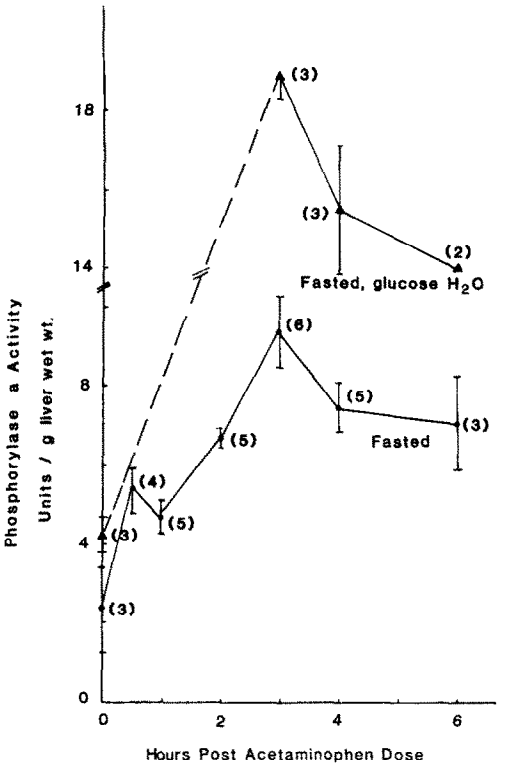

Fig. 1. Phosphorylase activation after acetaminophen *in vivo*. Male Sprague–Dawley rats were given 2 g/kg acetaminophen (see Methods) and fasted overnight with or without 5% glucose water to maintain liver glycogen. Homogenates of freeze-clamped liver samples obtained from animals at various times after dose were assayed in duplicate for phosphorylase *a*. Control activities (plotted as zero time values) were measured in samples actually obtained 1–6 hr after gavage with vehicle minus acetaminophen. With one exception where the value is the average of two determinations, data are means ± SE; N values (numbers of individual animals tested) are shown in the figure.

RESULTS AND DISCUSSION

Phosphorylase *a* was measured in samples of liver tissue obtained by a very rapid freeze-clamp procedure at selected times after dosing of 3-methylcholanthrene-induced, fasted Sprague–Dawley rats with acetaminophen (2 g/kg). Using the same animal model, we demonstrated previously a profound time-dependent depression of the Ca^{2+} accumulation activity of liver plasma membrane vesicles isolated 45 min to 2.5 hr post-dose [1] and, in addition, a significant decrease in Ca^{2+}-dependent plasma membrane ATPase activity [12]. The expected result of this functional deficit *in vivo* would be an increase in intracellular free [Ca^{2+}], which should lead to activation of phosphorylase *b* kinase and, in turn, to the conversion of phosphorylase *b* to the much more active phosphorylase *a* [13]. Consistent with the scheme is the observation that glycogen becomes depleted after acetaminophen dose [14].

Figure 1 shows that phosphorylase *a* activity increased markedly after acetaminophen, reaching a peak at 3 hr and remaining substantially elevated versus control liver values for at least 6 hr. The control activity plotted at time zero is the mean of determinations on samples (N = 3) of control livers obtained 1–6 hr after gavage with vehicle alone. Phosphorylase exhibits a circadian rhythm such that its activity increases to maximum at a time coinciding with 6–8 hr after dose in these experiments; however, the variation in control activities was small compared to the rise in activity observed after the hepatotoxic drug was administered. When rats were fasted but provided with 5% glucose water to help maintain liver glycogen but allow hepatic GSH to decline, a requirement for acetaminophen toxicity in the Sprague–Dawley rat, similar results were obtained (Fig. 1). However, maximum activation of phosphorylase (at 3 hr) was significantly greater in the rats provided with glucose water than in the fasted rats ($P < 0.01$).

When measurements of phosphorylase *a* were

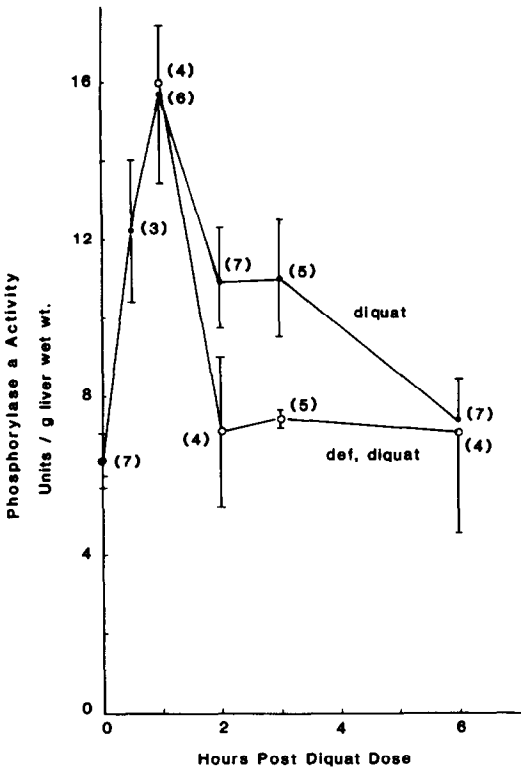

Fig. 2. Phosphorylase activation after diquat *in vivo*. Fed male Fischer-344 rats were given 0.1 mmol/kg diquat, and freeze-clamped liver samples were obtained and assayed at various times after dose as in Fig. 1. Control activities were measured in samples obtained 1–6 hr after vehicle. When used, desferrioxamine (0.24 mmol/kg, i.p., in saline) was injected 30 min prior to diquat to suppress Fe^{2+}-dependent pathways of lipid peroxidation. Data are means ± SE; N values are shown in the figure.

made in liver samples obtained from fed male Fischer-344 rats after diquat administration (0.1 mmol/kg), a marked elevation of phosphorylase was observed at 1 hr post-dose, followed by a decline (Fig. 2). However, activity remained substantially elevated until falling to a value not significantly different from controls at 6 hr. The early increase in phosphorylase correlates well with the time course of increased ethane and pentane expiration [14] and detection of 11-, 12-, and 15-hydroxyeicosatetraenoates, specific liver membrane lipid peroxidation products [6], in the same animal model. At 1 hr post-dose, inhibition of liver microsomal Ca^{2+} accumulation activity and increased microsomal permeability were also well established (Tsokos-Kuhn JO, unpublished results). These data seem to suggest that membrane lipid peroxidation induced by reactive oxygen species derived from diquat redox cycling [6] may produce an altered distribution of Ca^{2+} across the endoplasmic reticulum membranes, resulting in an increase in cytosolic free Ca^{2+}.

Fe^{2+} catalyzes the cleavage of H_2O_2 to produce hydroxyl radical ($OH^{\cdot}$), the most potent of the reactive oxygen species, and also promotes the beta-scission of lipid hydroperoxides to yield ethane or pentane and the corresponding lipid aldehydes. It has been demonstrated that iron plays a role in the lipid peroxidation and hepatic necrosis induced by diquat *in vivo* [15]. Superoxide generated by diquat redox cycling may increase the availability of reactive free iron species by reduction of ferric iron bound in ferritin. Both liver damage and ethane and pentane expiration are increased in iron-supplemented rats dosed with diquat and, conversely, both are decreased when animals are pretreated with desferrioxamine, an iron chelator [15]. Desferrioxamine has been shown also to inhibit lipid peroxidation *in vitro* [16, 17].

Figure 2 shows that pretreatment with desferrioxamine (0.24 mmol/kg 30 min prior to diquat) had no effect on the early peak of phosphorylase activity, even though such pretreatment has been shown to decrease significantly ethane and pentane expiration at 1 hr and decrease liver damage [15]. On the other hand, the phosphorylase activity declined abruptly to a level significantly lower ($P < 0.05$) than in the absence of desferrioxamine at 3 hr and remained at control level through 6 hr. It is unclear why the phosphorylase activity, and presumably cytosolic free Ca^{2+}, were elevated as much at 1 hr in the presence of desferrioxamine as in its absence. A direct role of lipid peroxidation in the initial rise of free Ca^{2+} may be called into question by these data. However, the deactivation of phosphorylase *a*, requiring a decrease of free Ca^{2+} toward normal levels, may be delayed or prevented by membrane lipid peroxidation. It may be that a *sustained* elevation of free Ca^{2+} is necessary for damage to occur.

Figure 3 demonstrates the time course of phosphorylase activation following administration of CCl_4 (2 ml/kg) to fed or fasted male Sprague–Dawley rats. There was an early rise in phosphorylase activity peaking near 1 hr, followed by a decline toward control level much as seen with diquat (Fig. 2). Subsequently, however, the activity underwent an additional substantial increase and appeared likely to continue to increase after 7 hr, although this was not tested. The early increase correlates well with the burst of lipid peroxidation that peaks at 30 min to 1 hr after CCl_4 [18], and with the similar time dependence of the effects of CCl_4 on plasma membrane and microsomal Ca^{2+} permeability (Figs. 4 and 5). Plasma membrane (Fig. 4) and microsomes (Fig. 5) exhibited marked increases in Ca^{2+} permeability demonstrable as early as 30 min after dose. Not only did the rate of Ca^{2+} loss from the vesicles increase during the time course of the efflux measurements, but the amount of Ca^{2+} associated with the vesicles at zero time declined as a function of time after the CCl_4 dose. The latter is also indicative of an increase in permeability, as previously shown for plasma membranes isolated 4 hr after CCl_4 dose [2]. The secondary increase in phosphorylase (Fig. 3) and presumably free Ca^{2+} coincides with the accumulation of total liver calcium demonstrated in early work on CCl_4 by Reynolds [19].

These data confirm the observation of Long and Moore [20] that phosphorylase is activated after CCl_4 *in vivo*. These findings may well be the result of the destruction by CCl_4 of the endoplasmic reticulum Ca^{2+} pump [11], as well as the increased permeability

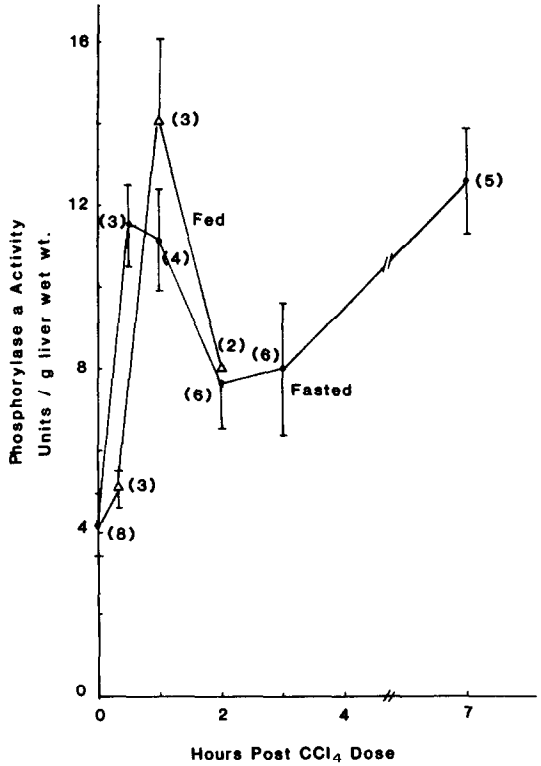

Fig. 3. Phosphorylase activation after CCl_4 *in vivo*. Male Sprague–Dawley rats, fed or fasted, were given 2 ml/kg CCl_4 (i.p., 1:1 in corn oil). Freeze-clamped liver samples were obtained at indicated times after dose and assayed as in Fig. 1. Control activities were measured as in Fig. 1. With one exception where the value is the average of two determinations, data are means ± SE of N values shown in the figure.

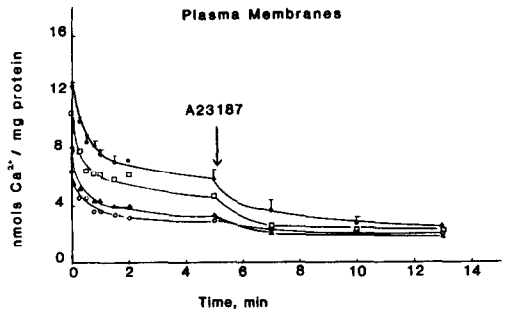

Fig. 4. Passive calcium efflux from control and CCl_4 plasma membrane vesicles. Membranes were isolated 30, 60 or 90 min after a dose of CCl_4 (2 ml/kg) and passively loaded at 0° for 18 hr in the presence of 1 mM $CaCl_2$ labeled with $^{45}Ca^{2+}$, as described [10]. Efflux of $^{45}Ca^{2+}$ was initiated by diluting a portion of the loading mixture into ice-cold isotonic buffered KCl medium containing 2 mM EGTA (ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid). At 5 min the ionophore A23187 (2 μM) was added to discharge remaining intravesicular Ca^{2+}. Key: (●) control membranes, mean ± SE (N = 3 separate preparations); (□) CCl_4, 30 min; (▲) CCl_4, 60 min; and (○) CCl_4, 90 min. Each of the CCl_4 curves was derived from the means of determinations on two separate preparations.

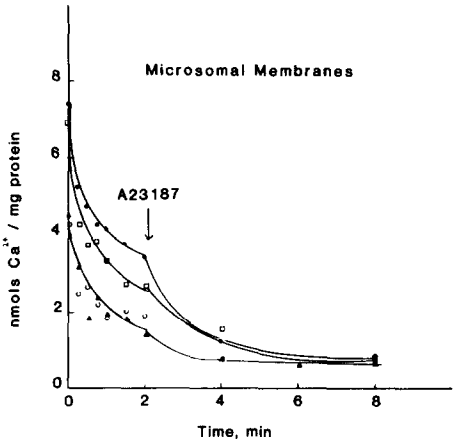

Fig. 5. Passive calcium efflux from control and CCl_4 microsomal vesicles. Experiments were as described in the legend to Fig. 4, except for the use of 2 hr instead of 18 hr passive loading. Symbols are defined in the legend of Fig. 4. Each curve was derived from the means of determinations on two separate preparations.

of both the endoplasmic reticulum and plasma membranes [2] to Ca^{2+}. Feeding of the animals had little effect on the results (Fig. 3), at least at the early times tested, consistent with the fact that fasting to deplete liver glutathione (GSH) is not required for CCl_4 toxicity, as it is for acetaminophen toxicity in the Sprague–Dawley rat.

All three agents tested produced 2.5-fold or greater increases in phosphorylase *a* activity versus controls, suggesting an activation of phosphorylase in the liver *in vivo* during exposure to hepatotoxic doses of these agents. Fasted or fed state did not alter the time courses of activation, indicating no major interference by glucagon-mediated cyclic AMP tone. It was shown previously by Long and Moore [20] with phosphorylase activated *in vivo* after CCl_4 that cyclic AMP levels do not change appreciably during the time course of activation, suggesting no general "stress" effect mediated by cyclic AMP in hepatotoxicity. Thus, an elevation of intracellular free Ca^{2+}, activating phosphorylase *b* kinase, is the most likely cause of the increased phosphorylase activity after these toxins.

The time course of acetaminophen-induced phosphorylase activation strongly resembles the time course of acetaminophen covalent binding [21] and appearance of histologic evidence of centrilobular hepatic necrosis *in vivo* [22]. Similarly, the time courses of CCl_4- and diquat-induced phosphorylase activation correlate well with observations of very early increases in measures of lipid peroxidation [15, 18] and microsomal Ca^{2+} pump inhibition [11]. Indeed, the early rise and fall, and then later rise of apparent intracellular free Ca^{2+}, as indicated by the changes in phosphorylase *a* activity, resemble strongly data of a classic study reporting the time course of changes in total liver calcium after CCl_4 [19].

Significantly, the administration of desferrioxamine prior to diquat, although having no effect on the early rise of phosphorylase *a* activity, prevented

the continued activation observed in its absence. It was demonstrated recently that increased iron availability during diquat intoxication potentiates both hepatic damage and membrane lipid peroxidation, while, conversely, chelation of iron by desferrioxamine protects against hepatic damage and lipid peroxidation [15]. The protective effect of desferrioxamine on the three parameters together is suggestive of a relationship between a sustained elevation of cytosolic Ca^{2+} and diquat hepatotoxicity *in vivo*. These data suggest further that the sustained elevation of Ca^{2+} levels in drug intoxication may play a fundamental role in the pathogenesis of toxic liver injury.

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REFERENCES

1. Tsokos-Kuhn JO, Todd EL, McMillin-Wood JB and Mitchell JR, ATP-dependent calcium uptake by rat liver plasma membrane vesicles: effect of alkylating hepatotoxins *in vivo*. *Mol Pharmacol* **28**: 56–61, 1985.
2. Tsokos-Kuhn JO, Smith CV, Mitchell JR, Tate CA and Entman ML, Evidence for increased membrane permeability of plasmalemmal vesicles from livers of CCl_4-intoxicated rats. *Mol Pharmacol* **30**: 444–451, 1986.
3. Tsokos-Kuhn JO, Smith CV, Hughes H and Mitchell JR, Liver membrane calcium transport in diquat-induced oxidative stress *in vivo*. *Mol Pharmacol* **34**: 209–214, 1988.
4. DiMonte D, Bellomo G, Thor H, Nicotera P and Orrenius S, Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch Biochem Biophys* **235**: 334–342, 1984.
5. DiMonte D, Ross D, Bellomo G, Eklow L and Orrenius S, Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. *Arch Biochem Biophys* **235**: 343–350, 1984.
6. Smith CV, Hughes H, Lauterburg BH and Mitchell JR, Oxidant stress and hepatic necrosis in rats treated with diquat. *J Pharmacol Exp Ther* **235**: 172–177, 1985.
7. Mitchell DB, Acosta D and Bruckner JV, Role of glutathione depletion in the cytotoxicity of acetaminophen in a primary culture of rat hepatocytes. *Toxicology* **37**: 127–146, 1985.
8. Gerson RJ, Cassini A, Gilfor D, Serroni A and Farber J, Oxygen-mediated cell injury in the killing of cultured hepatocytes by acetaminophen. *Biochem Biophys Res Commun* **126**: 1129–1137, 1985.
9. Smith CV and Mitchell JR, Acetaminophen hepatotoxicity *in vivo* is not accompanied by oxidative stress. *Biochem Biophys Res Commun* **133**: 329–336, 1985.
10. Tan AWH and Nuttall FQ, Characteristics of the dephosphorylated form of phosphorylase purified from rat liver and measurement of its activity in crude liver preparations. *Biochim Biophys Acta* **410**: 45–60, 1975.
11. Moore L, Davenport GR and Landon EJ, Calcium uptake of a rat liver microsomal subcellular fraction in response to *in vivo* administration of CCl_4. *J Biol Chem* **251**: 1197–1201, 1976.
12. Tsokos-Kuhn JO, Hughes H, Smith CV and Mitchell JR, Alkylation of the liver plasma membrane and inhibition of the (Ca^{2+}, Mg^{2+})-ATPase by acetaminophen. *Biochem Pharmacol* **37**: 2125–2131, 1988.
13. Hems DA and Whitton PD, Control of hepatic glycogenolysis. *Physiol Rev* **60**: 1–50, 1980.
14. Hinson JA, Mays JB and Cameron AM, Acetaminophen-induced hepatic glycogen depletion and hyperglycemia in mice. *Biochem Pharmacol* **32**: 1979–1988, 1983.
15. Smith CV, Evidence for the participation of lipid peroxidation and iron in diquat-induced hepatic necrosis *in vivo*. *Mol Pharmacol* **32**: 417–422, 1987.
16. Morehouse LA, Thomas CE and Aust SD, Superoxide generation by NADPH–cytochrome P-450 reductase: the effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. *Arch Biochem Biophys* **232**: 366–377, 1984.
17. Thomas CE, Morehouse LA and Aust SD, Ferritin and superoxide-dependent lipid peroxidation. *J Biol Chem* **260**: 3275–3280, 1985.
18. Hughes H, Smith CV, Tsokos-Kuhn JO and Mitchell JR, Quantitation of lipid peroxidation products by gas chromatography–mass spectrometry. *Anal Biochem* **152**: 107–112, 1986.
19. Reynolds ES, Liver parenchymal cell injury. II. Cytochemical events concerned with mitochondrial dysfunction following poisoning with carbon tetrachloride. *Lab Invest* **13**: 1457–1470, 1964.
20. Long RM and Moore L, Inhibition of liver endoplasmic reticulum calcium pump by CCl_4 and release of a sequestered calcium pool. *Biochem Pharmacol* **35**: 4131–4137, 1986.
21. Jollow DJ, Mitchell JR, Potter WZ, Davis DC, Gillette JR and Brodie BB, Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. *J Pharmacol Exp Ther* **187**: 195–202, 1973.
22. Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR and Brodie BB, Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* **187**: 185–194, 1973.