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# PARACETAMOL-INDUCED STIMULATION OF GLYCOGENOLYSIS IN ISOLATED MOUSE HEPATOCYTES IS NOT DIRECTLY ASSOCIATED WITH CELL DEATH

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**Abstract**—Paracetamol intoxication *in vivo* is known to be accompanied by depletion of hepatic glycogen stores. We have demonstrated a dose-dependent stimulation of glycogenolysis by paracetamol in glycogen-rich hepatocytes isolated from the mouse. Concentrations of paracetamol that produced plasma membrane damage were also found to activate glycogen phosphorylase *a* and deplete cellular glycogen contents. However, paracetamol-mediated stimulation of glycogenolysis could be dissociated from the events associated with paracetamol-induced cell killing. Both *N*-acetylcysteine and 2,4-dichloro-6-phenylphenoxyethylamine markedly reduced the extent of hepatocellular plasma membrane damage induced by paracetamol, yet neither agent prevented the activation of phosphorylase *a* nor the depletion of glycogen. These findings suggest that the hepatic glycogen depletion that accompanies paracetamol intoxication *in vivo* is due, at least in part, to a direct effect of the drug on the liver.

A variety of pathological and xenobiotic-induced toxic states are accompanied by enhanced rates of hepatic glycogenolysis. For example, diminished liver glycogen levels are observed following such events as hypoxia and ischemia [1, 2] as well as following exposure to biological toxins such as Ochratoxin A [3] and Aflatoxin $B_1$ [4]. Acceleration of hepatic glycogenolysis also occurs as a consequence of exposure to a diverse group of exogenous chemicals, including methotrexate [5], triazine [6], paraquat [7], carbon tetrachloride [8] and paracetamol [9].

Since accelerated glycogenolysis is a characteristic of such a diverse range of toxicities, it is possible that it may be associated with the mechanism of toxicity of these compounds. However, whether enhanced glycogenolysis plays a role in, or is a consequence of, the events leading to the manifestation of irreversible cytotoxicity has yet to be demonstrated. In the present study, we have examined the role of enhanced hepatic glycogenolysis in the events associated with the toxicity of the analgesic drug, paracetamol (acetaminophen) in order to determine if it is associated with paracetamol toxicity.

The mechanism whereby paracetamol stimulates glycogen degradation in the liver is not understood [9]. It is possible that the acceleration of glycogenolysis may occur via a direct effect of paracetamol upon the liver, either as the unchanged parent drug, or via a metabolite formed in the hepatocyte. Alternatively, the stimulation of glycogenolysis may be indirectly mediated via extrahepatic stimuli evoked by paracetamol intoxication. Such extrahepatic stimuli could include stress-induced increases in hepatic glycogen degradation, or alternatively, it may occur as a result of the hypothermia that accompanies paracetamol intoxication [10]. The use of an isolated hepatocyte model in this study enables us to distinguish between a direct and an indirect mechanism of paracetamol-induced stimulation of hepatic glycogenolysis. In an *in vitro* model, any extra-hepatic factors that may contribute to the stimulation of glycogenolysis by paracetamol are removed, allowing observation of the direct effect of the drug upon glycogen metabolism in the liver cell.

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## MATERIALS AND METHODS

*Materials*. Male Swiss mice (body weight 25–30 g) were obtained from Animal Resources Centre (Murdoch, W.A.). Collagenase (Worthington Type II) and RPMI 1640 culture medium were purchased from Flow Labs Australia (North Ryde, N.S.W.). Glucose-1-phosphate (disodium salt) and glycogen (from mollusc) were from Boehringer Mannheim (Sydney, N.S.W.). Paracetamol, glycylglycine, pyruvic acid, anthrone and thiourea were all obtained from Sigma Chemical Co. (St Louis, MO).

*Hepatocyte isolations*. Hepatocytes were prepared by collagenase perfusion of the liver as described previously [11] with the modification that the perfusion buffers were supplemented with 10 mM pyruvate and 60 mM glucose in order to prevent the depletion of glycogen that occurs during cell isolation in the absence of these substrates [12]. After isolation cells were resuspended into RMPI 1640 culture medium (supplemented with 0.1 mg/ml streptomycin, 100 U/ml penicillin and 10 mM HEPES, pH 7.4) and 3 ml aliquots were added to 60 mm collagen-coated culture dishes. The culture media was also supplemented with 50 mM glucose to activate glycogen synthase [13]. The cells were maintained in an incubator at 37° in a humidified 95% air:5% $CO_2$ atmosphere for 4 hr to allow cell attachment and equilibration. The cells were then washed three times with 3 ml phosphate buffered saline to remove unattached cells, before fresh culture medium, with or without paracetamol and protective agents, was added. The paracetamol and protective agents were

Table 1. Glycogen levels in mouse hepatocytes prepared and maintained with and without glucose supplementation of isolation and culture media

| Time (hr) | No glucose supplementation (N = 5) | 50 mM glucose supplementation (N = 6) |
|---|---|---|
| 0.5 | 47 ± 12* | 226 ± 43 |
| 1.0 | 42 ± 12 | 188 ± 18 |
| 2.0 | 31 ± 10 | 183 ± 25 |
| 3.0 | 30 ± 7.5 | 169 ± 16 |
| 4.0 | 24 ± 6.4 | 143 ± 11 |

* Glycogen contents are expressed as μg/mg protein. Values are mean ± SE. The *in vivo* liver glycogen content in these mice was 240 ± 3 μg/mg protein.

dissolved directly in the culture medium.

*Assay procedures.* Hepatocyte glycogen levels were determined using the anthrone reagent procedure of Carrol *et al.* [14] as described by others [9]. Briefly, the cells were washed 2 × 3 ml with phosphate buffered saline, then 2.0 ml of 3% perchloric acid was added to the cells and the plates were stored overnight at 4°. The cells were then scraped into centrifuge tubes and centrifuged for 15 min at 2000 *g*. A 0.2 ml aliquot of the resultant supernatant was assayed directly for glycogen content [9]. Glycogen phosphorylase *a* activity was determined in the hepatocytes as described by Bellomo *et al.* [15]. In brief, the cells were washed once with ice-cold isotonic saline and then 2 ml homogenisation medium containing 100 mM NaF, 20 mM EDTA, 0.5% (w/v) glycogen and 50 mM glycylglycine (pH 7.4) was added to the plate. The cells were sonicated with 2 × 10 sec bursts (Branson Sonifier Cell Disruptor B15, setting 1). A 0.2 ml aliquot of the resultant homogenate was incubated with an equal volume of a solution containing 100 mM glucose-1-phosphate, 2% (w/v) glycogen, 0.3 M NaF and 1 mM caffeine (pH 6.1) for 60 min. A reaction blank containing homogenisation buffer but not cells was run simultaneously. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid followed by 2.1 ml water. After centrifugation (2000 *g* × 15 min, 4°), a 0.5 ml aliquot of supernatant was assayed for inorganic phosphate using the molybdate–phosphate complex method [16]. For the assessment of plasma membrane damage, lactate dehydrogenase (LDH)† activity was determined in a 70 μl aliquot of cell-free supernatant in comparison to the total activity measured after cell lysis with Triton X-100 (0.5%) as detailed previously [11]. LDH was measured using an automated procedure (Roche COBAS BIO centrifugal analyser). Protein was estimated using the method of Hartree [17].

*Statistical analysis.* Two-way analysis of variance was performed using the computer package Genstat (Rothamsted Experimental Station, U.K.). If a significant variance ratio was indicated, differences between individual groups were investigated using the Newman–Keul's test or the Dunnett's test [18].

† Abbreviations: DPEA, 2,4-dichloro-6-phenylphenoxyethylamine; LDH, lactate dehydrogenase; NAC, *N*-acetylcysteine.

## RESULTS

### *Maintenance of glycogen content in isolated hepatocytes*

Basal concentrations of glycogen in hepatocytes isolated without glucose supplementation to the isolation buffers were low, presumably due to extensive glycogenolysis during cell isolation (Table 1). Inclusion of glucose and pyruvate during cell isolation along with glucose-supplementation (50 mM) of culture media resulted in a 4.5- to 6-fold increase in the glycogen content of the hepatocytes in comparison to those prepared without added glucose

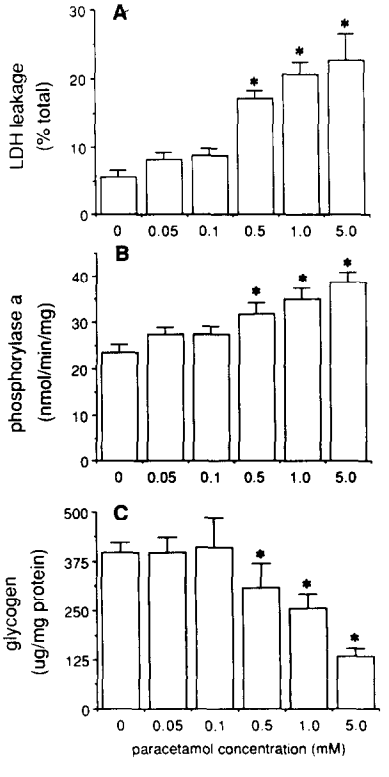


Fig. 1. The effect of paracetamol concentration on LDH leakage (A), glycogen phosphorylase *a* activity (B), and glycogen content (C) in hepatocytes after either an 8 hr (A and C) or a 90 min (B) incubation. Values are mean ± SE of five–six experiments. * Significant difference from controls (zero concentration), Dunnett's test. $P < 0.05$.

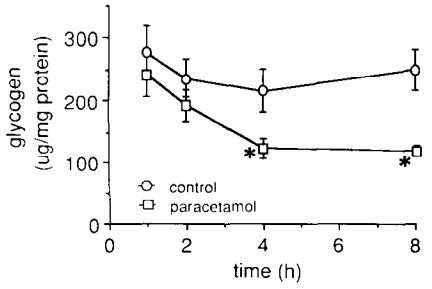


Fig. 2. The effect of paracetamol (5.0 mM) on glycogen contents in hepatocytes over an 8 hr incubation. Values are mean ± SE of 10 experiments. * Significant difference from controls (zero concentration), Newman–Keul's test, $P < 0.05$.

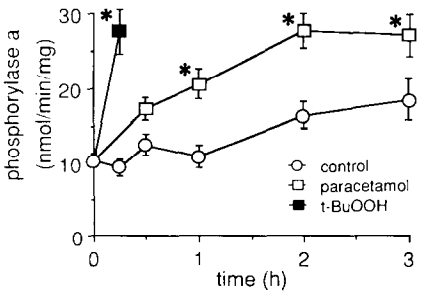


Fig. 3. The effect of paracetamol (5.0 mM) and *t*-butylhydroperoxide (0.5 mM) on glycogen phosphorylase *a* activity in isolated hepatocytes. Values are mean ± SE of nine experiments. * Significant difference from controls (zero concentration), Newman–Keul's test, $P < 0.05$.

(Table 1). We found that paracetamol had no effect on either cellular glycogen contents or phosphorylase *a* activity in cells prepared and maintained without glucose supplementation (data not shown). Consequently, all experiments were conducted in glycogen-rich hepatocytes. The toxic response to paracetamol in glycogen-depleted hepatocytes was essentially the same as in glycogen-rich hepatocytes (data not shown).

*Effect of paracetamol on cell viability and glycogenolysis: effect of paracetamol concentration*

The effect of paracetamol concentration on cell viability was determined after an 8 hr incubation. The 0.05 and 0.1 mM concentrations were not toxic, but concentrations of 0.5 mM and above produced significant LDH leakage (Fig. 1A). The effect of paracetamol concentration on glycogen phosphorylase *a* activity after a 90 min exposure is shown in Fig. 1B. No effect of 0.05 and 0.1 mM paracetamol on phosphorylase *a* activity was observed. However, the 0.5, 1.0 and 5.0 mM concentrations increased phosphorylase *a* activity by 34, 49 and 64%, respectively (Fig. 1B). A similar dose–response to paracetamol was observed in cellular glycogen contents after an 8.0 hr exposure (Fig. 1C). No effect of paracetamol at or below 0.1 mM was observed, whereas 0.5, 1.0 and 5.0 mM concentrations decreased cell glycogen contents by 22, 36 and 66%, respectively. Hence the effects of paracetamol upon glycogen metabolism are observed with concentrations that coincide with those that reduce the integrity of the plasma membrane.

*Paracetamol-induced activation of glycogenolysis in hepatocytes: the effect of time*

The time-course of glycogen depletion was followed using a concentration of 5.0 mM paracetamol (Fig. 2). A loss of hepatocyte glycogen did not occur until after 2.0 hr. Losses of 43 and 52% were observed at 4.0 and 8.0 hr, respectively (Fig. 2).The time-course of the effect of 5.0 mM paracetamol upon phosphorylase *a* activity is shown in Fig. 3. The presence of 5 mM paracetamol resulted in a 90% increase in phosphorylase *a* activity over controls at 1.0 hr. Phosphorylase *a* activity in paracetamol-poisoned cells remained elevated over control values after 2.0 and 3.0 hr incubation (Fig. 3). The presence of *t*-butyl hydroperoxide (0.5 mM), an agent known to induce phosphorylase *a* activation secondary to a

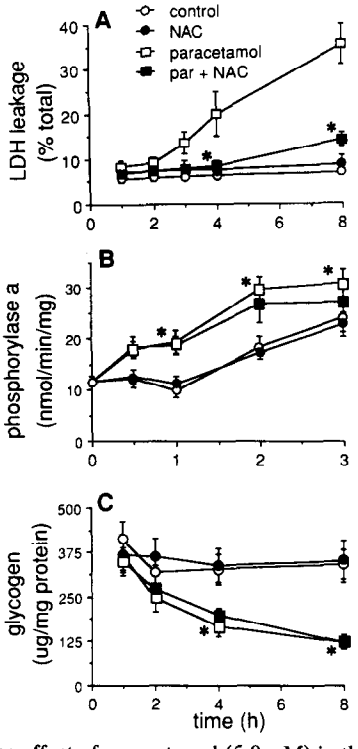


Fig. 4. The effect of paracetamol (5.0 mM) in the presence and absence of *N*-acetylcysteine on LDH leakage (A), glycogen phosphorylase *a* activity (B) and glycogen contents (C) in hepatocytes over either a 8.0 hr (A and C) or a 3.0 hr (B) incubation period. Values are mean ± SE of four–six experiments. In A (*) indicates that paracetamol + NAC (closed squares) is significantly different to paracetamol alone (open squares) (Newman–Keul's test, $P < 0.05$). In (B) and (C) (*) indicates that both paracetamol alone (open squares) and paracetamol + NAC (closed squares) are different from control values, yet not different from each other (Newman–Keul's test, $P < 0.05$).

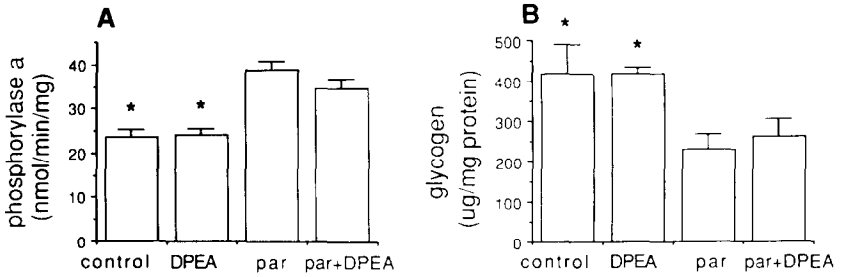


Fig. 5. The effect of paracetamol (5.0 mM) in the presence and absence of DPEA (50 μM) on (A) glycogen phosphorylase *a* activity in isolated hepatocytes after a 90 min incubation, and (B) glycogen contents after a 4.0 hr incubation. Values are mean ± SE of four experiments. * Significant difference from paracetamol values (Dunnett's test, $P < 0.05$).

perturbation of intracellular calcium homeostasis [15] (used here as a positive control) induced a 3-fold elevation in phosphorylase *a* activity (Fig. 3).

*The effect of hepatoprotective agents on paracetamol-induced activation of glycogenolysis*

In other experiments we examined the effect of *N*-acetylcysteine (NAC) on paracetamol-induced activation of glycogenolysis in isolated hepatocytes. The presence of 5.0 mM NAC protected against the loss of plasma membrane integrity that is associated with paracetamol intoxication (Fig. 4A). The extent of LDH leakage from hepatocytes incubated in the presence of 5.0 mM paracetamol for 8.0 hr was reduced by 79% in the presence of NAC (Fig. 4A). NAC has been shown previously to reduce the extent of covalent binding and the toxicity of paracetamol in this mouse hepatocyte model [19]. However, the presence of NAC did not prevent the activation of phosphorylase *a* by paracetamol in liver cells (Fig. 4B). Similarly, NAC did not prevent the paracetamol-induced depletion of cellular glycogen contents (Fig. 4C). The effect of the cytochrome P-450 inhibitor, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA), on paracetamol-mediated plasma membrane damage was also examined. The presence of 50 μM DPEA reduced the extent of LDH leakage after 8.0 hr exposure of hepatocytes to 5 mM paracetamol by 76%. However, the presence of DPEA did not prevent the activation of phosphorylase *a* induced during a 90 min exposure to paracetamol (Fig. 5A), nor did not prevent the loss of glycogen after a 4.0 hr incubation period (Fig. 5B). These findings suggest that the stimulation of glycogenolysis accompanying exposure to 5 mM paracetamol in isolated hepatocytes is not directly associated with paracetamol toxicity. Both NAC and DPEA markedly reduced toxicity without affecting glycogenolysis, indicating that although enhanced glycogenolysis coincides with the development of toxicity in paracetamol-poisoned liver cells, it can be dissociated from the toxic events that result in cell death.

## DISCUSSION

In general, standard procedures used in the preparation and incubation of isolated hepatocytes utilise buffers which do not contain high concentrations of glucose or gluconeogenic precursor substrates. This results in preparations with low glycogen levels due to extensive glycogenolysis in the cells during isolation and subsequent incubation procedures [12]. High concentrations of glucose, in the range of 50–70 mM [20] or gluconeogenic substrates such as glutamine, fructose or dihydroxyacetone [21, 22] are required to achieve measurable rates of glycogen synthesis, possibly via glucose-6-phosphate-mediated activation of glycogen synthase [13, 21]. In the present study it was desirable that the isolated hepatocyte preparation used had levels of glycogen that approximate the levels found *in vivo*. We found that supplementation of isolation and culture media with glucose significantly increased the glycogen contents of the isolated liver cells (Table 1). This allowed investigation of the effects of paracetamol upon hepatocellular glycogen metabolism in an *in vitro* system in which the cellular glycogen contents were maintained at levels that approximate those found *in vivo*.

The ability of high doses of paracetamol to reduce hepatic glycogen stores has been recognised previously. Centrilobular glycogen depletion has been observed to precede the development of cell necrosis in both paracetamol-poisoned rats and mice [23–25]. The loss of hepatic glycogen induced by paracetamol in the mouse has been shown to be dose-dependent [9], and results in the induction of hyperglycaemia [26]. However, the mechanism whereby paracetamol induces hepatic glycogenolysis is not understood [9].

Xenobiotic-induced acceleration of hepatic glycogenolysis may result from either indirect activation via extra-hepatic stimuli, or following direct interaction of the xenobiotic with the liver cell. Extra-hepatic factors will undoubtedly contribute to the loss of hepatic glycogen observed with paracetamol poisoning *in vivo*. For example, the marked centrally-mediated hypothermia that accompanies paracetamol intoxication in the mouse [10] could evoke a glycogenolytic response in storage tissues. Alternatively, paracetamol intoxication may result in the induction of a stress syndrome in the animal, increasing epinephrine release from the adrenal medulla, thereby activating hepatic glycogenolysis [1, 27]. In this study we have demonstrated that paracetamol can directly activate glycogenolysis in isolated hepatocytes. Paracetamol increased phosphorylase *a*

activity and decreased cellular glycogen levels in a dose-dependent manner. Both these parameters are considered useful measures of glycogenolysis [1]. Thus a component of the glycogenolysis observed *in vivo* appears to result from a direct effect of paracetamol on the hepatocyte. Such a mixture of direct and indirect effects on glycogenolysis has been demonstrated during $CCl_4$ intoxication [8, 28].

The finding that neither the protective thiol compound NAC, nor the cytochrome P-450 inhibitor DPEA [29], prevented the activation of glycogenolysis induced by paracetamol suggests that the reactive metabolite of paracetamol (*N*-acetyl-*p*-benzoquinoneimine) is not responsible for the acceleration of glycogen degradation in the paracetamol-poisoned liver cell. Both these agents reduced the toxicity associated with paracetamol exposure, either by enhancing the rate of detoxification of the reactive metabolite (as with NAC) or reducing the amount of reactive metabolite formed (as with DPEA). However, they did not prevent phosphorylase *a* activation or glycogen depletion. Thus it appears that it is paracetamol itself, or perhaps one of its nontoxic metabolites, that initiates glycogen breakdown.

A possible explanation of this glycogenolytic phenomenon could involve a mechanism similar to that induced by metabolic inhibitors such as dinitrophenol, carboxyactractyloside and amytal [30] or cyanide [31]. Respiratory toxins are thought to enhance rates of glycogenolysis by interfering with the phosphorylation–dephosphorylation equilibrium of the cell, resulting in an activation of phosphorylase *a* [31]. It is noteworthy that unchanged paracetamol has been shown to cause a reversible, dose-dependent rotenone-like inhibition of electron transport between NADH dehydrogenase and cytochrome *b* in isolated renal mitochondria [32]. It is likely that this action of paracetamol on mitochondrial respiration is not confined to the kidney alone since these respiratory processes are common to all aerobic tissues. We have confirmed these findings by exposing isolated mouse liver mitochondria to paracetamol (5 mM). A 20–25% decrease in uncoupler-released respiration with glutamate/malate as the energy substrate was observed (data not shown). Thus paracetamol itself may cause a reversible inhibition of hepatic mitochondrial respiration, resulting in an activation of glycogenolysis in a similar manner to that induced by the respiratory toxins as outlined above.

The dissociation of phosphorylase *a* activation from irreversible cell injury in paracetamol-poisoned cells (with NAC and DPEA) is an important finding. Phosphorylase *a* is considered a non-invasive indicator of cytosolic free calcium levels [33]. Hence phosphorylase *a* activity has been widely used to assess the importance of perturbations in intracellular calcium homeostasis in the development of irreversible cell injury in hepatocytes exposed to toxins such as menadione [34], *t*-butyl hydroperoxide [15], and *N*-acetyl-*p*-benzo-quinoneimine [35]. However, others have questioned the importance of the role of toxin-induced phosphorylase *a* activation in the development of irreversible cell damage as it was demonstrated that the loss of cell viability induced by an oxidative stress can be dissociated from changes in phosphorylase *a* activity [36]. The findings in the present study also suggest the dissociation of phosphorylase *a* activation from cell death in paracetamol-poisoned glycogen-rich hepatocytes. While NAC and DPEA both substantially reduced toxicity, neither prevented activation of phosphorylase *a* nor glycogen depletion.

Thus it appears a degree of caution should be exercised when implicating a role for altered intracellular calcium homeostasis in chemically-induced cell injury on the basis of phosphorylase *a* data alone. In addition, if phosphorylase *a* is to be used as an index of cytosolic free calcium levels in isolated hepatocytes, care should be exercised to ensure that the hepatocytes have adequate glycogen levels.

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