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DISSOCIATION OF CELL DEATH FROM COVALENT BINDING OF PARACETAMOL BY FLAVONES IN A HEPATOCYTE SYSTEM

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Abstract—Paracetamol metabolism and toxicity were studied in isolated rat hepatocytes. Cell damage, due to paracetamol, was shown to be dose dependent and was worse in cells from animals pre-treated with phenobarbitone. Exposure to 10 mM paracetamol for 1 hr caused a loss of intracellular reduced glutathione (GSH) and a later progressive leakage of isocitrate dehydrogenase (ICD). Treatment with (+)catechin, 3-O-methyl(+)catechin and promethazine reduced or prevented the paracetamol-induced ICD leakage. Similarly, studies on covalent binding of paracetamol showed that 3-O-methyl(+)catechin, which "protected" the cells, did so without affecting the amount of material bound covalently to cellular protein. Incubation in tissue culture for 24 hr, after prior treatment with paracetamol ± the protective agent, showed that the protected cells remained viable and attached to tissue culture plates much better than did the "unprotected" cells. These results suggest that the protective effect is much more than just a temporarily delayed cell death. GSH loss and covalent binding of paracetamol metabolites to cell protein are not sufficient causes of cell death, although they may act as starting points in the chain of events leading to cell death.

Paracetamol is a commonly used analgesic drug and when taken at the therapeutic dose, of around 1 g, is safe, but when taken in overdose causes centrilobular hepatic necrosis [1–3]. It has been shown that the toxic effect is accompanied by generation of a reactive metabolite, formed when paracetamol undergoes oxidative metabolism in the cytochrome P-450 system [3, 4]. Although the event of liver necrosis, due to paracetamol overdose, is well documented and widely recognised, the mechanism of cell injury for this and other hepatotoxins is a subject of controversy. Covalent binding of the metabolite to proteins or DNA, lipid peroxidation, free radical generation and calcium entry are all postulated as underlying mechanisms and possibly converging common pathways in liver cell necrosis [5]. The work of Mitchell and co-workers [6] suggests that the reactive metabolite can combine with glutathione (GSH) to form a harmless product, and that only when GSH is depleted does the metabolite cause cell injury. This naturally leads to procedures for treatment of the early stages of paracetamol overdose, designed either to block paracetamol metabolism or else to increase the synthesis of GSH or other sulphydryl compounds. The commonly used treatments are now methionine, *N*-acetylcysteine or cysteamine [7, 8]. However, these can only be effective if given before paracetamol metabolism is complete. In human studies it seems that therapy must be given within 8–10 hr of overdosage if it is to have any protective effect [9]. It is of interest that essentially all of the paracetamol is metabolised within 24 hr of overdose in man and within 5 hr in rat, but that evidence of liver cell necrosis is delayed until about 72 hr in man [2] and 8–12 hr in the rat. This suggests that paracetamol metabolism and the immediate covalent binding of metabolites to some essential cell macromolecules can only be the beginning of the process leading to cell death.

Our studies have concentrated on the events following after paracetamol metabolism and have used isolated rat hepatocytes to separate the events of death of these cells from circulatory, hormonal or other events in the whole animal. As a starting point we have used our previous work, using rat liver slices, in which we observed protection against paracetamol injury by addition of the flavone(+)catechin and the phenothiazine chlorpromazine [10]. Our objective has been to work towards an understanding of cell injury, which will permit protection of the liver cells, even though therapy is delayed until a late time when paracetamol metabolism is complete.

MATERIALS AND METHODS

All chemicals used were of the highest grade available and were obtained from BDH Ltd (Poole, U.K.), unless otherwise stated. Paracetamol and sterile 35% solution of bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (London, U.K.). Collagenase and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Boehringer Mannheim (Isleworth, U.K.). Promethazine was obtained from May and Baker Ltd. (Dagenham, U.K.); (+)catechin and 3-O-methyl(+)catechin were gifts from Zyma (Nyon, Switzerland). Vitamin E (DL-α tocopherol acetate) was a gift from Roche Products Ltd. (London, U.K.).

Isolation of hepatocytes. Adult male Wistar rats weighing 180–250 g were fed 41B pellet diet (Oxoid Ltd.) and allowed 0.1% solution of sodium phenobarbitone in distilled water, as drinking source, *ad libitum*. Phenobarbitone treatment was continued for a minimum period of 6 days and on the day before sacrifice, the animals were given Vitamin E (5 mg in 2 drops olive oil) orally. Hepatocytes were prepared according to the technique previously described by Seglen [11] and modified by Paine *et al.* [12]. The procedure was further modified as follows; the final cell suspension was subjected to an additional filtration through 56 μm pore size nylon gauge and the cells were not rolled in a rotary evaporator. The final washed cell pellet, composed of chiefly parenchymal cells with a consistent viability of 93 ± 3% (mean ± 1 S.D. of 12 perfusions) assessed by trypan blue exclusion.

Incubation of hepatocytes. The cells were resuspended into Hepes–Ringer containing 25 mg/ml albumin (HRA), as described by McLean and Nuttall [10], at approximately 300 mg wet weight cells/ml solution. One ml of the cell suspension was added to 4.0 ml of HRA solution in silanised 25 ml Erlenmeyer flasks and incubated at 37° in a shaking water bath, set at 80 strokes/min, under oxygen. After 1 hr in 10 mM paracetamol, the cells were washed by centrifugation at 5 *g* for 3 min. The cell pellet was resuspended in 5.0 ml fresh HRA, without any paracetamol and the cells were washed once again. The final washed cell pellet was again suspended in fresh HRA solution and incubated further; 0.5 ml aliquots were sampled at regular intervals over a period of 4 hr.

Measurement of isocitrate dehydrogenase (ICD) activity and cellular reduced glutathione (GSH). ICD activity in both the supernatant (medium) and the homogenised cell pellet was estimated as previously described by McLean and McLean [13] and cellular GSH was measured according to Beutler *et al.* [14].

Measurement of covalent binding. [^{14}C]-Paracetamol (Amersham International Limited, Amersham, U.K.) labelled in ring C-3 and -5 positions and of radiochemical purity assessed to be ≥99.9% after purification by a high performance liquid chromatography (HPLC) technique as described by Devalia and McLean [15] was used.

Cells in HRA solution were treated with approximately 1.0 μCi of [^{14}C]-paracetamol, diluted to a final 10 mM concentration with unlabelled paracetamol. Half ml samples were collected, and the cells immediately washed, free of albumin, by mixing with 9.5 ml cold 0.15 M saline, followed by centrifugation at 1500 *g* for 3 min. Each cell pellet was resuspended in 10.0 ml 10% (w/v aqueous) trichloroacetic acid (TCA). The precipitated cellular proteins were washed three times by re-suspension and centrifugation in 10.0 ml 10% TCA. The washed TCA insoluble pellet was washed twice more with 10.0 ml 80% (v/v aqueous) methanol. The final washed protein pellet was suspended in 2.0 ml 1 M NaOH for digestion at 45° overnight. Radioactivity in 1.0 ml of the digested pellet was determined by liquid scintillation counting in a Packard Tri-Carb System. Protein in the pellet digest was determined by the method of Lowry *et al.* [16].

Tissue culture of hepatocytes. All cells to be grown in tissue culture were initially washed twice, by suspension and centrifugation (5 *g* for 3 min) in sterile Williams medium E (Gibco Bio-Cult, Uxbridge, U.K.), containing 4 mM glutamine and 100 units and 100 μg per ml of benzylpenicillin BP and streptomycin sulphate BP (Glaxo, Greenford, U.K.) respectively (termed supplemented medium E). Following suspension in fresh supplemented medium E, containing 5% foetal calf serum (FCS, Gibco), the cells were seeded into tissue culture plates (Nunclon, Gibco) and incubated for 22–24 hr at 37° in a Leec humidified incubator.

Measurement of DNA in cells grown in tissue culture. At the end of the tissue culture period, the DNA content of the cells floating in the culture medium and the cells attached to the plates, was determined as described by Richards [17], using the modification of Abraham *et al.* [18].

RESULTS

Effect of brief exposure to paracetamol on cell viability and GSH concentration and prevention of cell leakage by some protective agents

When cells of high viability are added to HRA solution, a small amount of enzyme activity is found in the supernatant within 5 min (around 5–10% of the total). Over the next 5 hr there is a gradual leak of ICD and increase in number of dead cells, permeable to trypan blue. Figure 1 shows that cells treated with 10 mM paracetamol for 30 min leak ICD at 2.5 hr and 4.5 hr. There is no paracetamol effect in the first ½ hr of incubation. Table 1 shows that leakage

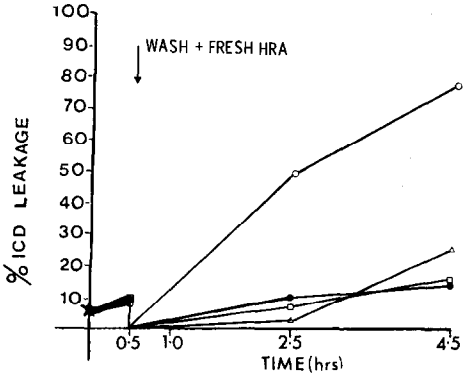

Fig. 1. Effect of 1.0 mM (+)catechin on paracetamol toxicity. Cells from a phenobarbitone pre-treated rat were incubated in HRA solution, pH 7.5 at 37° ± 10 mM paracetamol. After 0.5 hr incubation all the cells were washed free of paracetamol as described. Incubation was continued for up to 4.5 hr, in absence of paracetamol, and 0.5 ml samples taken for analysis of ICD activity. 1.0 mM (+)catechin was added at time zero, of incubation, and maintained after washing or else added only after 0.5 hr paracetamol exposure to the washed, resuspended cells. Neither (+)catechin nor paracetamol altered the total ICD activity present in the supernatant and cells. (●—●) Control cells; (○—○) paracetamol treated cells; (□—□) paracetamol and (+)catechin (pre- and post-wash) treated cells; (△—△) paracetamol and (+)catechin (post-wash only) treated cells.

Table 1. Effect of 30 min paracetamol treatment on enzyme leakage from hepatocytes isolated from stock and phenobarbital pre-treated rats

Treatment during incubation in from: Hepes–Ringer–albumin for 30 min	Per cent ICD leakage at 4 hr in cells	
	Stock	PB pre-treated
HRA	10	6
HRA + 2 mM paracetamol	8	22
HRA + 4 mM paracetamol	—	39
HRA + 5 mM paracetamol	12	54
HRA + 8 mM paracetamol	—	54
HRA + 10 mM paracetamol	19	53
HRA + 20 mM paracetamol	24	—

Cells were incubated as described in the Methods section.

Samples of total cell suspensions, from incubation flasks, were collected and per cent leak was expressed as:

$$\left(\frac{\text{ICD activity in incubation medium}}{\text{ICD activity in medium + ICD activity in cells}}\right) \times 100.$$

Results are expressed as means of two separate experiments.

is dose dependent and is far greater in cells from phenobarbital treated rats, which were used in all the subsequent experiments.

Table 2 shows that the ICD leakage and loss of cell viability after paracetamol treatment can be prevented to a considerable extent by the presence of 0.35 mM 3-O-methyl(+)catechin during the whole incubation period, or even when 3-O-methyl-(+)catechin is added after a 1 hr paracetamol exposure has ended. Half hour exposure to paracetamol led to similar but reduced cell damage, which was also preventable by 3-O-methyl(+)catechin. Figure 1 shows extensive protection by (+)catechin after 30 min exposure to paracetamol. As a rule (+)catechin was less effective as a protective agent than 3-O-methyl(+)catechin. Promethazine did not show a significant protective effect when results from four separate experiments were pooled and compared with pooled results from twelve paracetamol treated cell incubates. When the results were compared by the paired *t* statistic, however, protection was shown to be highly significant ($P < 0.005$).

3-O-Methyl(+)catechin was found to be the most effective of the compounds studied, with respect to its hepatoprotective properties, and for this reason was studied further.

Treatment of hepatocytes with paracetamol for 1 hr leads to a rapid depletion of cellular GSH, with little recovery in the following few hours. 3-O-Methyl(+)catechin effectively prevents

Table 2. Effect of some protective agents on paracetamol-induced ICD leakage and loss of cell viability

Treatment during incubation for: 0–1 hr	1–4 hr	Per cent ICD leakage at 4 hr incubation	Per cent viability at 4 hr incubation
HRA	HRA	12.1 ± 1.8 (4)	88.2 ± 5.8 (7)
HRA, 10 mM paracetamol	HRA	67.1 ± 17.5 (12)	38.7 ± 21.4 (15)
HRA, 10 mM paracetamol, 0.35 mM 3-O-methyl-(+)catechin	HRA 0.35 mM 3-O-methyl-(+)catechin	21.1 ± 7.5 (5)	81.8 ± 7.7 (6)
HRA, 10 mM paracetamol	HRA, 0.35 mM 3-O-methyl-(+)catechin	23.0 ± 8.5 (3)	85.7 ± 7.1 (3)
HRA, 10 mM paracetamol, 1 mM (+)catechin	HRA,1 mM (+)catechin	32.1 ± 7.7 (6)	70.6 ± 11.7(5)
HRA, 10 mM paracetamol, 50 μM promethazine	HRA, 50 μM promethazine	52.6 ± 18.4 (4)	43.0 ± 9.3 (4)

Hepatocytes were incubated as described in Methods section.

Viability was measured by trypan blue exclusion.

Results are expressed as means ± S.D. for a number (shown in brackets) of experiments carried out on separate days (i.e. pooled results).

In testing the signficance of the effects, Student's *t* test was applied to the paired results for paracetamol alone and paracetamol + protective drug, measured on the same day, thereby taking into account the large "between experiments" variance. By this test all four treatments reduced ICD leakage, with a P value < 0.005.

Neither 3-O-methyl(+)catechin, (+)catechin nor promethazine altered the total ICD values in control incubations.

Table 3. Effect of paracetamol ± 3-O-methyl(+)catechin treatment on glutathione (GSH) concentration in hepatocytes

Time of incubation (hr)	Concentration of GSH (nmole/mg protein) in cells after exposure to: No treatment for 1.0 hr	Paracetamol for 1.0 hr	Paracetamol for 1.0 hr + 3-O-methyl(+)catechin throughout
0	41	41	41
0.5	31	20	20
1.0	33	14	14
2.0	23	9	10
3.0	25	10	11
4.0	27	11	14

Hepatocytes were incubated in HRA, either in the presence or absence of 10 mM paracetamol ± 0.35 mM 3-O-methyl(+)catechin, for 1.0 hr before being washed. Further incubation was carried out in paracetamol-free HRA with 3-O-methyl(+)catechin and ½ ml samples collected and centrifuged. GSH was analysed in the cell pellets as described.

Results are expressed as means of two separate experiments.

GSH content of the original liver is about 35–45 nmoles/mg protein.

paracetamol-induced ICD leakage, but does not prevent the paracetamol induced GSH loss (Table 3).

Covalent binding of [^{14}C]*-paracetamol*

When purified [^{14}C]-paracetamol was added to the incubation mixture, ^{14}C-material was found to bind tightly to the cells, in about the same quantities as were found after hepatotoxic dosage *in vivo* [6]. The amounts bound were dependent on the time of incubation with paracetamol and remained constant during subsequent washing and reincubation and were unaffected by addition of 3-O-methyl(+)catechin (Fig. 2).

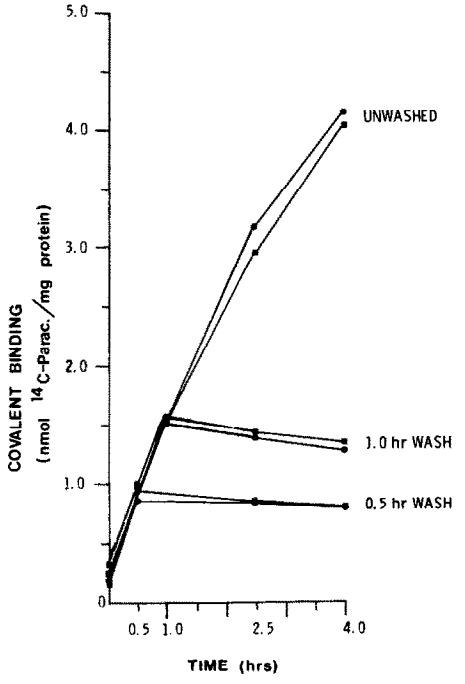

Fig. 2. Effect of 3-O-methyl(+)catechin on covalent binding of paracetamol. Cells isolated from a phenobarbital pre-treated rat were incubated in HRA in 10 mM [^{14}C]-paracetamol (sp. act. 1.0 μCi in 50 μmoles) ± 3-O-methyl(+)catechin. Cells were washed after 0.5 hr or 1.0 hr or else were unwashed for 4.0 hr. Half ml aliquots were collected and analysed for covalent binding, as described in Methods. (●—●) Paracetamol treated cells; (■—■) paracetamol and 3-O-methyl(+)catechin treated cells.

The fate of exposed cells in tissue culture

At the end of the 4 hr incubation, in control HRA solution, the hepatocytes were not only viable as noted by trypan blue exclusion, but also retained the ability to adhere to plastic culture dishes and survive for 24 hr. During this time the cells made colonies consisting of cords of cells. About 60% of the added cells adhered and survived.

Many of the cells exposed to paracetamol for 1 hr were capable of adhering to culture dishes, but they failed to form colonies and to a large extent the paracetamol treated cells ended up as non-viable floating material in the medium. Protection by addition of 3-O-methyl(+)catechin for 4 hr incubation was followed by high viability for the next 24 hr of cell culture (Table 4).

DISCUSSION

The experiments we have described show that the isolated hepatocytes respond to paracetamol in a way that is analagous to the behaviour of liver cells *in vivo*. The injury is made worse by phenobarbitone pre-treatment, is dose dependent and cell death follows a latent period [1–3, 6].

It is well documented that metabolism of paracetamol via the cytochrome P-450 system leads to the generation of some highly reactive metabolite and is accompanied by a depletion of intracellular GSH. After a large dose of paracetamol, GSH depletion can reach 80–90% and is followed by covalent binding of paracetamol, to hepatocellular proteins [6].

Previous studies have shown that cells can be protected against cellular damage, by the addition of exogenous sulphur-containing amino acids [19, 20], which act presumably by increasing the cel-

Table 4. Survival of hepatocytes in tissue culture, after exposure to paracetamol ± 3-O-methyl(+)catechin

Treatment during incubation	Per cent 24 hr survival of treated cells
1. Control	59.1 ± 16.2
2. + 10 mM Paracetamol	13.7 ± 5.2
3. + 10 mM Paracetamol ± 0.35 mM 3-O-methyl(+)catechin	35.4 ± 11.3

Hepatocytes were initially incubated in HRA for 1.0 hr, under the incubation conditions tabulated, then washed free of paracetamol and taken up in fresh HRA ± 3-O-methyl(+)catechin, before subsequent incubation for 4 hr. At the end of the 4 hr incubation period the cells were washed with sterile medium and grown in tissue culture for a further period of 24 hr.

Survival at 24 hr is expressed as:

$$\left(\frac{\text{DNA in cells attached to tissue culture plates}}{\text{DNA in attached cells + DNA in cells floating in medium}}\right) \times 100.$$

Results are expressed as per cent means ± S.D. for three separate experiments. Student's t test was employed to assess statistical significance of the difference between pooled values.

$P < 0.01$ (2:1).
$P < 0.05$ (3:2).
Not significant (3:1).

lular GSH concentration, or by other —SH containing trapping agents. The GSH depleting effect of paracetamol in the present studies is similar to that shown by various authors [21, 22]. In the present studies, it has been observed that 3-O-methyl(+)catechin neither increases GSH concentration nor prevents its depletion, but clearly protects the cells against damage as assessed by enzyme leakage, dye exclusion and viability in tissue culture. The observation that protection, by the flavones, is afforded even when these agents are added after paracetamol metabolism has ended and that 3-O-methyl(+)catechin protects without affecting covalent binding of paracetamol suggests that these compounds are acting by a mechanism other than by preventing metabolism to—and binding of—the reactive metabolite.

Labadarios *et al.* [22] found that α-mercaptopropionylglycine also protects without altering covalent binding, in contrast to cysteamine which prevents binding [23]. Consideration of the shape of the covalent binding curve (Fig. 2) suggests that the extent of covalent binding to hepatocellular proteins, is a cumulative process and that this process may be terminated by simply washing out the paracetamol, without preventing cell necrosis in the following hours of incubation. Also, since the "protected" cells can be made to survive for at least 24 hr in tissue culture, this suggests that the protective effect is much more than just a temporarily delayed cell death.

Exactly how the protective agents act is a matter of speculation, but it seems possible that they have a specific stabilising effect on the cell membrane and/or other vital cellular macromolecules, such as the lysosomal enzymes. What seems certain, however, is that GSH depletion and covalent binding of paracetamol are not sufficient causes of cell death. Presumably, they may be triggers that lead to a sequence of events, in a lethal process, which can be blocked by the flavones.

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