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LIPID PEROXIDATION, PROTEIN SYNTHESIS, AND PROTECTION BY CALCIUM EDTA IN PARACETAMOL INJURY TO ISOLATED HEPATOCYTES

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Abstract—Hepatocytes from rats treated with phenobarbitone were exposed to 10 mM paracetamol for 1 hr and then incubated in buffered Ringer solution. Enzyme leakage and trypan blue entry became severe in the paracetamol treated cells some 2 hr after the end of exposure. These signs of cell injury could be blocked by 4 mM CaEDTA added during or after paracetamol exposure. CaEDTA did not alter covalent binding of [^{14}C]paracetamol. Ca^{2+} free media did not prevent paracetamol injury. Lipid peroxidation was observed in cells but could be blocked without protecting the cells. Protein synthesis was depressed early on in cells previously exposed to paracetamol, CaEDTA did not protect against this inhibition. These observations suggest that an early cytoplasmic lesion develops into a later lethal lesion at the cell surface.

Paracetamol is hepatotoxic to man and animals when given in overdose. While an initial conversion to a reactive metabolite which binds to cell macromolecules is well described [1, 2], the subsequent processes leading to liver cell necrosis are less well understood. The later stages of cell injury are particularly important because a number of patients who have taken overdoses of paracetamol come to hospital some 12–24 hr after the dose, when treatment with methionine or *N*-acetyl cysteine is probably no longer useful [3, 4]. At this time liver cell function is still relatively intact, but severe liver cell injury may develop over the course of the next 48 hr [1]. We have established a model system for this situation, using isolated hepatocytes exposed briefly to paracetamol and then incubated for the next 3 or 4 hr in the absence of paracetamol. In this system the hepatocytes develop signs of injury and death during the later stage of incubation, i.e. in the absence of free paracetamol. Cell injury can be measured by leakage of enzymes or development of permeability to trypan blue. We have previously shown that injury is dependent on the concentration of paracetamol in the 1-hr exposure stage and on pretreatment of the animals with phenobarbitone [5]. The injury can be blocked by a number of drugs such as (+)-catechin [6]. We have now observed that high concentrations of calcium EDTA protect the cells, while removal of the calcium from the incubation medium does not. We have used this observation to devise experiments to investigate the role of calcium and protein synthesis in the late stages of the cell injury produced by paracetamol.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest grade available and were from BDH Ltd. (Poole, U.K.) unless otherwise stated. Paracetamol, vitamin E (DL-tocopherol acetate), and sterile 35% solution of bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (London, U.K.). Collagenase and NADP were purchased from Boehringer Mannheim (Isleworth, U.K.). Calcium EDTA was obtained from Sinclair Pharmaceuticals Ltd. (Godalming, U.K.). HEPES (*N*-2-hydroxyethylpiperazine -*N*′-2-ethanesulphonic acid) was obtained from Gibco Europe (Uxbridge, U.K.). (Ring-3,5-^{14}C]paracetamol and L-[1-^{14}C]leucine were purchased from Amersham International Ltd. (Amersham, U.K.).

Animals. Adult male Wistar rats (A. Tuck, Essex, U.K.) weighing 180–250 g at time of perfusion were fed 41B pellet diet (Oxoid Ltd., London, U.K.) and allowed 0.1% solution of sodium phenobarbitone in tap water, as the drinking source *ad libitum*. Phenobarbitone treatment was continued for a minimum period of 5 days. On the day before sacrifice the animals were given an extra dose of oral vitamin E (5 mg in 2 drops olive oil) in order to maintain viability and ion transport capacity of isolated tissues, since they are impaired in minor and transient vitamin E deficiency states [7, 8].

Isolation of hepatocytes. Hepatocytes were prepared by collagenase perfusion of the liver, according to the technique previously described by Devalia *et al.* [6]. The final washed cell suspension was composed chiefly of parenchymal cells which had an average viability of $90 \pm 3\%$ (mean ± 1 S.D., $N = 14$) as assessed by trypan blue exclusion.

Incubation of hepatocytes. The cells were resuspended in HEPES buffered Ringer containing 25 mg/ml albumin, as described by McLean and Nuttall [5], and fortified with 0.1 mM final concentration of each of 22 amino acids [9] (HRA). The Mg^{2+} concentration in HRA is 1.2 mM and Ca^{2+} was varied from 0 to 4 mM. One ml of the cell suspension containing approximately $10.7 \times 10^6 \pm 2.5 \times 10^6$ cells/ml, was added to 4.0 ml of HRA solution in silanised 25 ml Erlenmeyer flasks and incubated at

37° in a shaking waterbath, 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 at room temperature. The loose cell pellet was resuspended in 5 ml fresh HRA, without paracetamol and centrifuged again. The final washed cell pellet was suspended in fresh HRA solution and incubated further. Samples (1 ml) were taken at intervals and rapidly centrifuged at 1200 *g* for 2 min. The supernatant (medium) was removed and the cell pellet resuspended in 1 ml of distilled water and dispersed by vortex mixing.

Measurement of isocitrate dehydrogenase (NADP$^+$), EC 1.1.42 (ICD). ICD activity in both supernatant (medium) and the resuspended cell pellet was estimated using a semi-automatic spectrophotometric system (Pye Unicam SP8-100 Rate Rack) at 25°. Leakage of enzyme from cells into the medium was expressed as percentage of total activity, i.e. % leakage = (ICD activity in medium/ICD in medium + ICD in pellet) × 100.

Measurement of covalent binding. Purified [^{14}C]-paracetamol was used, after removal of impurities by the HPLC technique of Devalia and McLean [10]. Cells in HRA solution were treated with approximately 2.0 μCi of [^{14}C]paracetamol, diluted to a final 10 mM concentration with unlabelled paracetamol and incubated as above. Samples (0.5 ml) were collected and covalent binding estimated as described by Devalia *et al.* [6].

Measurement of lipid peroxidation. Samples (1.5 ml) were taken at the end of the incubation and assayed for malondialdehyde using the thiobarbituric acid reaction (TBA) as described by Ernster and Nordenbrand [11], using 156 as the mM extinction coefficient for the malondialdehyde complex.

Measurement of protein synthesis. After pre-treatment with paracetamol for 1 hr, the cells were washed and resuspended in HRA solution containing the usual amino acid mixture (that is, containing 0.1 mM DL-leucine). They were reincubated for 10 min and then approximately 0.25 μCi (5 nmoles) of L-[1-^{14}C]leucine was added to each flask. Samples (0.5 ml) were collected and incorporation of L-[1-^{14}C]leucine was determined as described by Judah and Nicholls [9].

RESULTS

Table 1 shows that 1 hr exposure to 10 mM paracetamol followed by removal of paracetamol leads to a slight leakage of enzyme activity after 2 hr incubation. There is a marked leakage at 4 hr, which becomes even more severe at 5 hr. At this time cell viability has dropped to about half and lipid peroxidation, measured as thiobarbituric acid reactive material, is greatly increased in the paracetamol treated cells. There is considerable variation in the magnitude of these effects between experiments but the pattern is consistent and variation within experiments using the same batch of hepatocytes is small in comparison with the large effect of paracetamol.

Addition of 4 mM CaEDTA blocks ICD leakage, peroxidation and much of the loss of viability, whether the addition is made before or directly after the end of paracetamol exposure. However, when CaEDTA addition is delayed until 1 or 2 hr after removal of paracetamol then protection is reduced or absent. (The amount of EDTA present in the ICD assays was kept less than 0.5 mM, since concentrations greater than this inhibit the enzyme.)

The paracetamol injury is apparently blocked by CaEDTA at 2, 4 and 5 mM, but not 1 mM (Table 2), although lipid peroxidation as assessed by the TBA reaction is still blocked by 1 mM CaEDTA. The dose response curve varied between experiments so that in some, 1 mM CaEDTA protected and in others 2 mM CaEDTA was insufficient to protect.

Lipid peroxidation is a marked feature of the injured cells following removal of paracetamol, and

Table 1. The effect of subsequent addition of CaEDTA on cell damage caused by exposure to paracetamol for 1 hr

Treatment	Time from start of incubation ICD leakage % of total 2 hr	4 hr	5 hr	Lipid peroxidation (nmoles/10^7 cells at 5 hr)	% Viability at 5 hr
Control	3.6 ± 3.6	6.5 ± 2.5	8.1 ± 3.2	6.4 ± 3.8	86 ± 8.5
Paracetamol	5.4 ± 4.9	26.5 ± 10.2	37.2 ± 10.7	22.7 ± 16.0	49 ± 17
Paracetamol + CaEDTA (t0 hr)	1.0 ± 0.5	1.9 ± 2.0	6.2 ± 4.2	1.3 ± 0.5	72*
Paracetamol + CaEDTA (t1 hr)	4.2 ± 3.8	4.5 ± 2.7	9.5 ± 4.0	4.3 ± 3.5	71 ± 4.9
Paracetamol + CaEDTA (t2 hr)	5.1 ± 3.5	14.6 ± 8.6	17.6 ± 11.7	11.9 ± 7.6	66 ± 25.5
Paracetamol + CaEDTA (t3 hr)	7.6 ± 3.8	26.7 ± 10.1	32.7 ± 13.9	29.0 ± 13.8	34 ± 47.4

* 1 value only.

Paracetamol = 10 mM; CaEDTA = 4 mM; ICD = isocitrate dehydrogenase.

ICD results are expressed as mean ± 1 S.D. for at least 4 values from 2 separate experiments.

Cells from a phenobarbitone treated rat were incubated with paracetamol for 1 hr and then washed and resuspended as described in the Methods section.

CaEDTA was added to the normal HRA solution at the times indicated.

Total ICD in the samples (i.e. activity in cells + supernatant) remained essentially constant throughout the incubations and was not altered by paracetamol or EDTA.

The HRA contained 1 mM Ca^{2+} in all the incubation conditions.

Table 2. The effect of CaEDTA concentration on the cell damage following exposure to paracetamol for 1 hr

Treatment	Time from start of incubation 2 hr ICD leakage % of total	5 hr	Lipid peroxidation (nmoles/10^7 cells at 5 hr)
Control	4.3 ± 1.4	7.8 ± 4.3	3.5 ± 1.6
Paracetamol	8.2 ± 6.5	47.4 ± 17.0	25.7 ± 6.2
Paracetamol + CaEDTA (5 mM)	2.3 ± 1.9	6.7 ± 6.4	3.3 ± 1.2
Paracetamol + CaEDTA (4 mM)	1.7 ± 0.4	4.9 ± 1.8	1.9 ± 0.5
Paracetamol + CaEDTA (2 mM)	0.8 ± 1.1	4.5 ± 0.9	1.8 ± 0.7
Paracetamol + CaEDTA (1 mM)	3.5 ± 2.5	21.6 ± 17.2	5.1 ± 2.4

Paracetamol = 10 mM; ICD = isocitrate dehydrogenase.
All results mean ±1 S.D. for at least 3 values.
Cells from phenobarbitone treated rats were isolated and incubated with paracetamol for 1 hr and then resuspended as described in the section on methods. CaEDTA was present throughout the incubation time.
The HRA contained 1 mM Ca^{2+} in all the incubation conditions.

is greatly reduced by the presence of CaEDTA (Tables 1 and 2). We find that on continuous exposure to paracetamol throughout the incubation period lipid peroxidation is also reduced, but this does not prevent enzyme leakage taking place (Table 3). Presumably the anti-oxidant activity of paracetamol blocks lipid peroxidation.

With continuous exposure to paracetamol throughout the incubation time, CaEDTA has only a slight protective effect (Table 3).

The removal of extracellular calcium from the incubation medium leads to a marked increase in enzyme leakage from control hepatocytes (no paracetamol) (Fig. 1). Figure 1 also shows how addition of 1 mM or 4 mM Ca^{2+} to the Ringer solution prevents enzyme leakage from control cells, but not from paracetamol exposed cells, while 4 mM CaEDTA protects both control and paracetamol treated cells. (Since the Ringer solution contains 1.2 mM Mg^{2+} the addition of 4 mM CaEDTA results in a free Ca^{2+} release of 50 μM from the chelate as calculated using the stability constants of Martell and Smith [12].) Lipid peroxidation and cell viability measurements follow the same pattern as enzyme leakage. There is a marked increase of lipid peroxidation after 5 hr incubation in Ca^{2+} free medium, even without paracetamol, and this is blocked by addition of 1 mM Ca^{2+} (data not shown).

Figure 2 shows that protein synthesis as measured by incorporation of [^{14}C]leucine into cell protein is inhibited in cells pre-treated with paracetamol, and that CaEDTA, which powerfully protects the cells against loss of viability in these circumstances, does not prevent depression of protein synthesis.

Table 4 shows that CaEDTA does not affect covalent binding of paracetamol to cell proteins.

DISCUSSION

The experiments described here show that the early phase of cell injury by paracetamol comprising metabolism, glutathione depletion and covalent binding of paracetamol metabolites, is followed by a latent phase which leads, some hours later, to the last phase of cell death. Metabolism and covalent binding belong entirely to the first phase [13], which in these experiments ends with removal of paracetamol after 1 hr. At this time the cell injury pro-

Table 3. Absence of effect of CaEDTA on cell damage caused by continued exposure to paracetamol for 5 hr

Treatment	Time from start of incubation 2 hr ICD leakage % of total	5 hr	Lipid peroxidation (nmoles/10^7 cells at 5 hr)
Control	8.5 ± 3.1	12.7 ± 6.9	3.0 ± 1.9
Paracetamol	8.0 ± 1.7	42.6 ± 2.6	1.5 ± 0.1
Paracetamol + CaEDTA (4 mM)	9.5 ± 3.3	33.1 ± 7.2	1.6 ± 0.1

Paracetamol = 10 mM; CaEDTA = 4 mM; ICD = isocitrate dehydrogenase.
All results mean ±1 S.D. for at least 3 values.
Cells from phenobarbitone treated rats were isolated and incubated with paracetamol for the full 5 hr. CaEDTA was present throughout the incubation time.
The HRA contained 1 mM Ca^{2+} in all the incubation conditions.

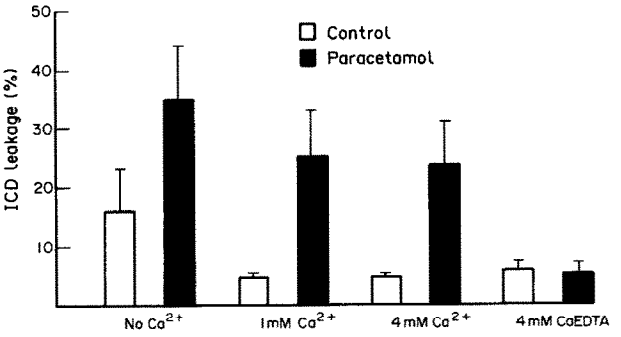

Fig. 1. Histogram showing the effect of Ca^{2+} on ICD leakage in isolated hepatocytes, from a phenobarbitone treated rat, exposed to paracetamol for 1 hr, then reincubated without paracetamol for a further 4 hr. ICD is expressed as % leakage of total at 5 hr.

cess is underway, but can be prevented from going to its completion in cell death, by CaEDTA or several other protective agents [6, 14, 15].

We find that considerable amounts of lipid peroxidation products are formed in injured cells (Tables 1 and 2) whether the injury is produced by exposure to a pulse of paracetamol or incubation in Ca^{2+} free media, or failure to give the hepatocyte donor extra vitamin E. Equally, lipid peroxidation can be blocked by low doses of EDTA [16] or continuous presence of the phenolic antioxidant paracetamol without preventing paracetamol injury and cell death. Evidently lipid peroxidation readily takes place in injured cells, may even make the injury worse, but cannot be regarded as a basic mechanism essential for injury in paracetamol poisoning. The TBA reaction has been used as a measure of lipid peroxidation in these experiments. Previously Recknagel and Ghoshal [16] found that EDTA blocked both malondialdehyde production and conjugated diene formation in a liver microsome preparation undergoing lipid peroxidation in the presence of ascorbic acid. Smith *et al.* [17] found good correlation between five different measures of lipid peroxidation in isolated hepatocytes, so that it seem likely that TBA reactive material gives an approximate measure of the attack on unsaturated lipids in hepatocytes, even though it is known that malondialdehyde is rapidly metabolised by mitochondria [16].

The role of calcium in paracetamol injury to hepatocytes is somewhat clarified by these experiments. External free calcium is not essential for paracetamol injury to hepatocytes, as we previously showed for paracetamol injury to liver slices [18].

Some 95% of cell Ca^{2+} is bound to mitochondria and endoplasmic reticulum, so there remains the possibility that shifts of ionised Ca^{2+}, into or out of cytoplasmic compartments may play a role in the latent phase, or the final phase of cell death [19, 20]. The mechanism by which CaEDTA protects the cells, preventing them from entering the last phase of cell death, is unknown. Chelation of heavy metal ions seems an unlikely mechanism of protection, since 0.1 mM EDTA would be more than adequate for this purpose. There seem to be two major possibilities. First, CaEDTA might enter the cytoplasmic compartment and act as a buffer for ionised Ca^{2+} in the physiological μmolar range. This would account for the requirements for relatively large amounts of CaEDTA (4 mM), since the dissociation constant is such that in order to buffer internal Ca^{2+} at around 0.5 μM concentrations in the presence of 20 mM Mg^{2+} an internal concentration of EDTA of 2.5 mM would be required (calculated using the stability constants of Martell and Smith [12]). However, EDTA complexes do not readily enter cells and it seems possible that the CaEDTA complex acts at the cell surface, and this prevents one of the late steps of the latent or final phases of cell injury. Neither CaEDTA nor protective agents such as +catechin will prevent the injury that follows 5 hr of continuous exposure to 10 mM paracetamol. It seems that this severe condition with high levels of covalent

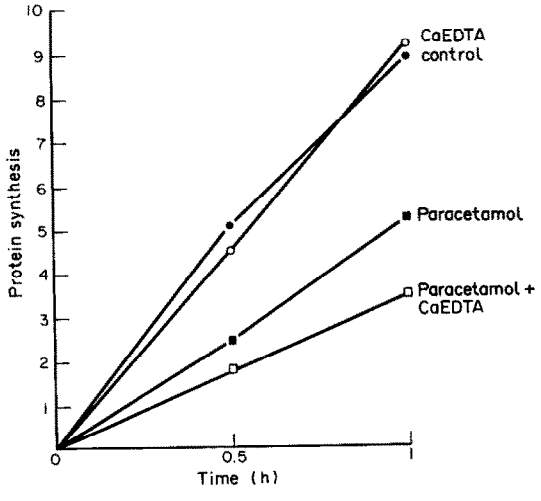

Fig. 2. The effect of 1 hr pretreatment with paracetamol and of CaEDTA on protein synthesis in isolated hepatocytes from a phenobarbitone treated rat. Protein synthesis was measured by incorporation of [^{14}C]leucine into cell protein and is expressed as nmoles [^{14}C]leucine/mg cell protein. The cells were pre-incubated with or without 10 mM paracetamol and with or without 4 mM CaEDTA for 1 hr and then re-isolated, suspended in HRA with or without the EDTA and incubated for 10 min and then [^{14}C] leucine added as described in the section on methods. The HRA contained 1 mM Ca^{2+} in all the incubation conditions.

Table 4. The effect of CaEDTA on covalent binding of [^{14}C]-paracetamol to hepatocytes during 3 hr of exposure

Treatment	Paracetamol bound/mg cell protein (nmoles)		
	Incubation time		
	1 hr	2 hr	3 hr
Paracetamol (10 mM)	1.2 ± 0.14	1.6 ± 0.1	2.5 ± 0.2
Paracetamol (10 mM) + CaEDTA (4 mM)	1.3 ± 0.3	1.9 ± 0.2	2.5 ± 0.2

Results are mean ± 1 S.D. N = 2.

Cells from a phenobarbitone treated rat were incubated with purified [^{14}C]-paracetamol (10 mM) for 3 hr and binding to cell proteins measured as described in the section on methods.

The blank value (T0) of 0.22 nmoles/mg protein was subtracted from all the values.

The HRA contained 1 mM Ca^{2+} in all the incubation conditions.

binding brings into play mechanisms of toxicity not amenable to block by CaEDTA. It is possible that these conditions also play a part in the injury process *in vivo*. So far we have not been able to protect animals against paracetamol injury by giving protective drugs in the latent period (i.e. 5 hr after intraperitoneal dosage when paracetamol metabolism is essentially complete).

Since EDTA binds Ca^{2+} with great avidity it is not possible to investigate the question whether some complex other than the Ca^{2+} form of EDTA would protect the cells except in Ca^{2+} and Mg^{2+} free media which themselves damage cells. The addition of CaEDTA in the presence of 1.2 mM Mg^{2+} will cause only small changes in free Ca^{2+} concentration through Ca^{2+}–Mg^{2+} exchange. However, this may be enough to account for the protective effect of CaEDTA on control cells incubated in Ca^{2+} free medium.

We observe that protein synthesis is impaired early on immediately after one hours exposure to paracetamol, and the covalent binding of metabolites is not prevented by CaEDTA. This again suggests that an early cytoplasmic injury is unaffected by CaEDTA. The early injury can then lead to the later lethal damage at the cell surface, as shown by leakage of enzymes and entry of dye. Perhaps CaEDTA can bind to the cell surface sites such as those controlling the exposed gap junctions of the isolated hepatocytes [21]. Inhibition of protein sythesis itself does not lead to cell injury, since cycloheximide block of protein synthesis does not lead to impaired viability (McLean and Beales, unpublished).

So far the picture of cell injury by paracetamol seems to be one of a multi-stage process with early cytoplasmic events setting in train a series of changes which culminate in cell surface damage and cell death. The sequence of events must be understood before we can control it.

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