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EVIDENCE FOR A DIRECT ROLE OF INTRACELLULAR CALCIUM IN PARACETAMOL TOXICITY

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Abstract—There is considerable evidence that an increase in cytosolic Ca^{2+} is involved in the cytotoxicity of a variety of agents. However, the direct demonstration of such involvement has proved difficult. In the present study, loading of freshly isolated hamster hepatocytes with the Ca^{2+} specific chelator Quin 2 (2-[(2-bis[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis-[carboxymethyl]amino-quinoline) provided significant protection against the loss of viability caused by paracetamol. This was evident both when the cells were co-incubated with Quin 2-AM and paracetamol, and when the cells were incubated with Quin 2-AM after prior exposure to paracetamol and its complete removal from the hepatocytes. These observations provide direct evidence that an increase in intracellular Ca^{2+} is the cause of cell death in hepatocytes exposed to paracetamol. Further, the fact that Quin 2 is protective even after some time suggests that, for alterations of cytosolic Ca^{2+} to be detrimental, they must be sustained. The effects of Quin 2 on plasma membrane blebbing of paracetamol-exposed hepatocytes were less pronounced than on cell viability. This is in contrast to the effects of the direct-acting thiol-reducing agent, dithiothreitol, which was equally effective in preventing blebbing and loss of viability. It is concluded that alterations of cytosolic Ca^{2+} are less directly linked to plasma membrane blebbing than to loss of cell viability.

Intracellular calcium plays multiple roles, essential to the control of cellular function. Recent studies have implicated an elevation of cytosolic Ca^{2+} in the final common pathway of cell death caused by a wide range of toxins, including paracetamol, through its putative reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NABQI‡) [1–6]. This elevation is thought to be due, at least in part, to oxidative stress [7]. NABQI, following the depletion of glutathione, causes oxidation of protein thiol groups, particularly those of membrane-bound Ca^{2+}-translocases [6]. Studies *in vitro* have shown that the resulting inactivation of the Ca^{2+}-translocases of both the endoplasmic reticulum (ER) and the plasma membrane (PM) is reversible by the addition of thiol-reducing compounds, such as glutathione and dithiothreitol (DTT) [8]. Further, the addition of DTT to isolated hepatocytes can reverse paracetamol-induced PM-blebbing and prevent loss of viability [9]. Thus, oxidation of thiol groups within Ca^{2+}-translocases causes impaired regulation of cytosolic Ca^{2+} levels, leading to an increase in the free concentration, which is strongly implicated as a cause of cell death in hepatocytes.

The normal cytosolic concentration of Ca^{2+} is approximately 10^{-7} M [10]. Ca^{2+}, at concentrations of 10^{-4} M, will activate Ca^{2+}-dependent proteases [11], nucleases [12], and phospholipases *in vitro* [13]. However, the concentration required for these effects in the intact cell remains unclear. It has been reported that inhibitors of the Ca^{2+}-dependent protease, calpain, such as leupeptin and antipain, prevent the toxicity of cystamine to isolated hepatocytes [14]. It has been suggested that elevation of cytosolic Ca^{2+} might also have profound effects on cytoskeletal elements [15, 16]. Villin is a possible target. At normal concentrations of Ca^{2+}, this component cross-links actin filaments into bundles, but when the concentration of Ca^{2+} is raised above 1 μM, as occurs in paracetamol toxicity [6], the bundles are dispersed and the filaments fragmented [17].

If an elevation of cytosolic Ca^{2+} does directly mediate the toxic effects of paracetamol, then it should be possible to prevent this by chelation of the cation. Several of the normal biochemical consequences of raised cytosolic Ca^{2+} can be successfully prevented in this way. For example, platelet aggregation caused by a variety of agonists is inhibited by prior loading of the cells with Quin 2 [18]. In the present study, the effects of the specific Ca^{2+}-chelator, Quin 2 [19], on the cytotoxic effects of paracetamol in isolated hepatocytes, both during and after their exposure to the toxin, were determined.

MATERIALS AND METHODS

Materials and isolation of hepatocytes. Hepatocytes were isolated from male Golden Syrian hamsters (90–110 g, from Belgrave Trading Supplies;

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‡ Abbreviations used: BME, basal medium Eagle's; DTT, dithiothreitol; EBSS, Earl's balanced salt solution; GSH, reduced form of glutathione; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]; NABQI, *N*-acetyl-*p*-benzoquinoneimine; Quin 2, 2-[(2-bis-[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis-[carboxymethyl]aminoquinoline; Quin 2-AM, 2-[(2-bis-[carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis-[carboxymethyl]aminoquinolinetetrakis-[acetoxymethyl]ester.

maintained on Labsure PRD diet), by the 3-step collagenase perfusion method of Tee *et al.* [9], and suspended in Ca^{2+}-free Earle's balanced salt solution (EBSS) with basal medium Eagle's (BME) vitamin and amino acid supplements. EBSS and BME vitamins were both purchased from Gibco (Uxbridge, U.K.). Cells were preincubated for 15 min at 37° in a shaking water bath, washed in fresh buffer, and kept on ice for up to 30 min before use. Incubation of hepatocytes was carried out in a shaking water bath at 37°, with an atmosphere of 95% O_2:5% CO_2. The pH of the medium was further stabilized by the inclusion of 10 mM HEPES buffer. Quin 2-AM (Sigma Chemical Co., Poole, U.K.) and [G^3H]-Quin 2-AM (411 MBq/mmol) (Amersham Radiochemical Co., Amersham, U.K.) were combined to give a specific activity of 1.67 MBq/mmol. The extent of Quin 2-AM loading of the cells was determined in all experiments, by removing 1-mL portions of the cell suspensions, washing three times and then subjecting the final suspension to liquid scintillation spectrometry. Ca^{2+}-free medium was used throughout, to avoid Quin 2-driven diffusion of Ca^{2+} into the cells.

Loading of cells with Quin 2-AM and incubation with paracetamol. Two different models of paracetamol toxicity were employed. In the first, the effect of co-incubation of cells with the chelating agent and paracetamol was investigated. The possible direct effect of Quin 2 on the oxidation of paracetamol was first determined, using the microsomal formation of 3-*S*-glutathionyl paracetamol, at 2 mM, as an index of activation. The method was as described previously [20]. At concentrations of up to 1 mM, Quin 2 caused a maximum decrease in the oxidation of paracetamol of less than 10% (data not shown). Hepatocyte suspensions, at 1×10^6 cells/mL, were incubated with 2 mM paracetamol, together with 100 μM Quin 2-AM, 1.5 mM DTT or no addition, for up to 285 min. At various times, 1-mL portions of the suspension were removed and either put on to ice for determination of Quin 2-AM loading, or added to glutaraldehyde (final concentration of 2.5%) for later assessment of viability using trypan blue, at a final concentration of 0.1%, and of plasma membrane blebbing [9]. In parallel, control experiments, paracetamol was omitted from the incubation.

In the second model of toxicity, the 2-phase model of Tee *et al.* [9], hepatocytes were preincubated with paracetamol, which was then removed by washing prior to the addition of Quin 2-AM. Thus, the effects of Ca^{2+}-chelation could be studied at a time when Ca^{2+} homeostasis was already disrupted [6]. Hepatocyte suspensions, at 1×10^6 cells/mL, were incubated with 2 mM paracetamol for 50 min. At the end of this time, although the cells showed morphological changes, there was no significant loss of viability. Parallel, control incubations were performed in which the paracetamol was omitted. The cells were then washed, to remove all of the paracetamol and its metabolites [9], resuspended and then portions of the suspension incubated with 200 μM Quin 2-AM, 1.5 mM DTT or no addition. Control cells were similarly treated. The cells were incubated for up to 90 min (phase II), during which time 1-mL portions of the suspension were removed for assessment of Quin 2-AM loading and hepatocyte viability and PM blebbing, as described above.

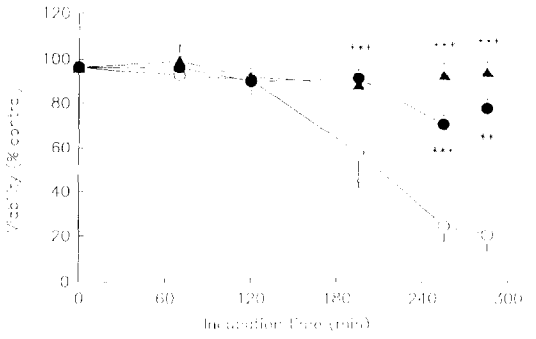

Fig. 1. Effect of concomitant addition of 100 μM Quin 2-AM (●) or 1.5 mM DTT (▲) on the cytotoxicity of 2 mM paracetamol in isolated hamster hepatocytes. Control cells (○) were incubated with paracetamol alone. Values are means ± SE from up to five different cell preparations. Where an error bar is not visible if falls within the symbol (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ c.f. control cells).

Analysis of the data. Cell viability and plasma membrane blebbing were both assessed, for each cell preparation, by counting two fields of 1 mm^2 [20]. The average of the two counts was then used to calculate the mean results for several cell preparations, each from a different animal. Comparisons were made by Student's *t*-test for paired samples. The null hypothesis was rejected at $P < 0.05$.

RESULTS

The proportion of freshly isolated hepatocytes which excluded trypan blue (viable cells) was 0.82. During incubation in buffer alone for 285 min, this gradually declined to 0.65. In all experiments, the viability of the cells has been expressed as a percentage of that of appropriate control cells incubated for a corresponding length of time.

Exposure of hamster hepatocytes to paracetamol at a concentration of 2 mM resulted in a progressive loss of viability after 120 min of incubation, with only 19% of the cells viable after 285 min (Fig. 1). There was a rapid increase in the number of viable cells exhibiting plasma membrane blebbing during incubation with paracetamol, which increased from 15% at the start of the experiment to 95% after 285 min (Fig. 2). The addition of DTT, 1.5 mM, as a positive control, prevented any significant loss of viability, throughout the entire incubation period, as expected from earlier work with this compound [9]. Viability after incubation for 285 min was 95% of the control value (NS). DTT also completely prevented the increase in the number of cells that became blebbed. Indeed, there was a modest reduction in the number of blebbed cells after 200 min incubation (Fig. 2).

The intracellular concentration of Quin 2 in cells incubated with the esterified chelating agent at 100 μM ranged from 1.16 mM to 1.96 mM. The loss of viability of cells co-incubated with paracetamol and Quin 2-AM was considerably less than with

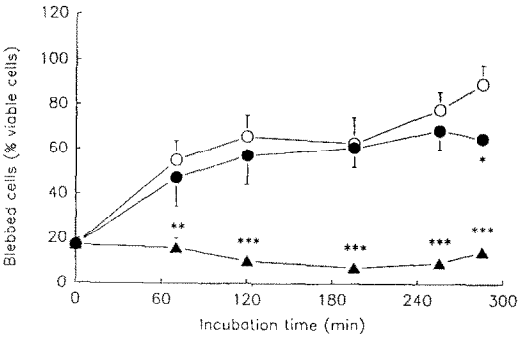

Fig. 2. Effect of concomitant addition of 100 μM Quin 2-AM (●) or 1.5 mM DTT (▲) on the plasma membrane blebbing induced by 2 mM paracetamol in isolated hamster hepatocytes. Control cells (○) were incubated with paracetamol alone. Values are means ± SE from up to five different cell preparations. Where an error bar is not visible it falls within the symbol (*$P < 0.05$; **$P < 0.02$; ***$P < 0.01$ c.f. control cells).

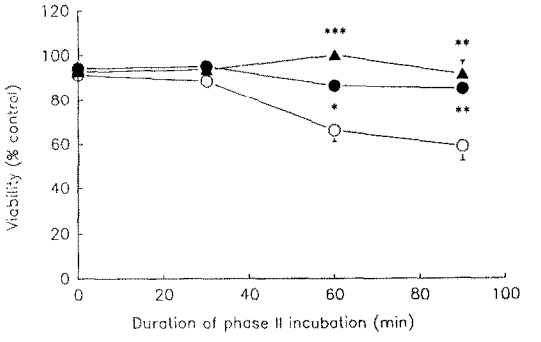

Fig. 3. Effect of 200 μM Quin 2-AM (●) and of 1.5 mM DTT (▲) on the cytotoxicity induced by prior exposure of isolated hepatocytes to 2 mM paracetamol for 50 min, following which all of the paracetamol was removed by washing. See text for details. Control cells (○) were incubated in buffer alone during phase II of the experiment. Values are means ± SE from three different cell preparations. Where an error bar is not visible it falls within the symbol (*$P < 0.05$; **$P < 0.02$; ***$P < 0.01$ c.f. control cells).

paracetamol alone (Fig. 1). After 285 min of incubation, viability was still 80% of control values. Although Quin 2-AM was able to attenuate the loss of viability of hepatocytes, it had very little effect on the number of viable cells showing plasma membrane blebbing (Fig. 2).

In the two-phase model of paracetamol toxicity, the proportion of control cells viable at the start of phase II was 0.75. Incubation of these cells in buffer alone resulted in a slight decrease in the proportion of cells which were viable, to 0.70 after 1.5 hr. The viability of cells exposed to paracetamol, 2 mM, for 50 min during phase I and then incubated in buffer alone during phase II, decreased from 92% of the control value at the start of the second phase to 60% after 1.5 hr (Fig. 3). This decrease in viability was accompanied by an increase in the percentage of blebbed cells, from 14% to 34% after 1.5 hr (Fig.

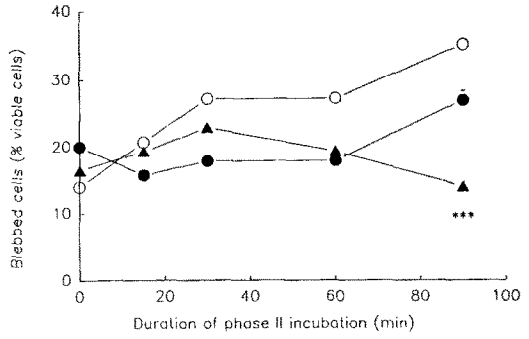

Fig. 4. Effect of 200 μM Quin 2-AM (●) and of 1.5 mM DTT (▲) on the plasma membrane blebbing induced by prior exposure of isolated hepatocytes to 2 mM paracetamol for 50 min, following which all of the paracetamol was removed by washing. See text for details. Control cells (○) were incubated in buffer alone during phase II of the experiment. Values are means from two to three different cell preparations (***$P < 0.01$ c.f. control cells).

4). Addition of DTT during phase II incubation maintained viability at >90% of control values, there being no significant deterioration in the viability of such cells over the 1.5 hr of phase II incubation (Fig. 3). Not only did DTT prevent any loss in the viability of cells exposed to paracetamol during phase I incubation, it also prevented and even slightly reduced the percentage of such cells showing blebbing of their plasma membrane, from 16% to 13% (Fig. 4). The quality of these cells was much better than that of the cells exposed only to paracetamol [9].

In hepatocytes incubated with 200 μM Quin 2-AM during phase II, the intracellular concentration of the chelator increased to >1.8 mM within 15 min and was maintained at that concentration thereafter. Quin 2 prevented the loss of viability of cells incubated with paracetamol during phase I. Viability only decreased from 92% to 80% of control values over this interval (Fig. 3). Plasma membrane blebbing in such cells increased roughly in parallel to that of cells exposed to paracetamol alone, and although there was a tendency for the Quin 2 treated cells to show less blebbing than the control cells, this did not reach stastistical significance (by ANOVA). The number of Quin 2 treated cells which were blebbed reached 27% after 1.5 hr, which was not significantly different from the percentage of blebbed cells following incubation in buffer alone during phase II (Fig. 4).

DISCUSSION

Paracetamol is cytotoxic to isolated hepatocytes, and this is widely believed to reflect the same underlying biochemical processes as those responsible for the necrosis produced by large doses of the drug *in vivo*. It has been shown that paracetamol is converted into a highly reactive electrophilic intermediate, NABQI [21]. This readily arylates cellular nucleophiles, including GSH and proteins [21]. Based on these observations, it was suggested that the toxicity of paracetamol was due to the arylation

of critical enzymes within the cell [22]. The discovery that the toxicity of paracetamol is due to the formation of a reactive metabolite that depletes hepatic stores of glutathione led to the introduction of successful antidotes for the treatment of paracetamol poisoning [23]. Many of these act as precursors of GSH [24]. This effect on the synthesis of GSH is also evident in isolated hepatocytes, which are protected from the cytotoxicity of paracetamol by agents such as methionine and *N*-acetylcysteine [25]. However, two observations led to this view of paracetamol toxicity being challenged. Firstly, it was found that NABQI is not only an electrophile but also a potent oxidizing agent [26]. Further, the early biochemical and morphological changes caused by paracetamol can be completely reversed by thiol-reducing agents [9]. Secondly, precursors of GSH, such as *N*-acetylcysteine, can prevent paracetamol toxicity to isolated hepatocytes even after all of the paracetamol has been removed [25]. These observations led to the proposal that paracetamol, through the formation of NABQI, is toxic due to the reversible oxidation of key thiol groups and that GSH, either endogenous or synthesized from thiol-containing antidotes, acts to reduce such disulphides [25, 27].

The implication of raised cytosolic Ca^{2+} in the cytotoxicity of a number of agents [1–6], and the demonstration that the enzymes involved can be reversibly inhibited by thiol-oxidizing agents [28], led to the proposal that the initial effects of cytotoxins such as paracetamol are due to the reversible disruption of Ca^{2+}-translocates by thiol group oxidation [6], and that antidotes, through the synthesis of GSH, reverse these effects by reduction of the disulphides thus formed [29]. If this is so, then it should be possible to delay and/or prevent toxicity by chelating all of the raised Ca^{2+} in the cytosol, even relatively late in the process, at times when thiol-containing antidotes are still effective [25]. Indeed, this was found in the present study, in which the Ca^{2+}-specific chelating agent, Quin 2, substantially attenuated the toxicity of paracetamol to isolated hepatocytes. This is true both when the cells are co-incubated with Quin 2-AM and paracetamol, and when the Quin 2-AM is added after removal of the paracetamol. In the former situation, Quin 2 does not act by inhibiting the activation of paracetamol, as shown by the lack of any direct effect on the oxidation of paracetamol *in vitro* and in the latter situation any effect on metabolism would not be relevant to the protective effects of the chelating agent. Thus, the likeliest explanation for the protective effect of Quin 2 is chelation of raised cytosolic Ca^{2+}. This provides a direct demonstration of the involvement of increased cytosolic Ca^{2+} in the toxicity of paracetamol, in the same way that the role of Ca^{2+} in platelet aggregation can be demonstrated [18].

Although Quin 2 prevents much of the loss of viability that occurs in cells exposed to paracetamol, it has much less of an effect on the plasma membrane blebbing of such cells. In contrast, and as shown previously by Tee *et al.* [9], the thiol-reducing agent, dithiothreitol, largely prevents the loss of viability and completely prevents the plasma membrane blebbing. Indeed, DTT can reverse blebbing that has already occurred. Thus, the loss of viability (as indicated by the uptake of trypan blue) caused by paracetamol appears not to be a direct consequence of plasma membrane blebbing. A similar dissociation between loss of viability and plasma membrane blebbing has been shown previously [30]. Whereas loss of viability appears to be a direct consequence of raised cytosolic Ca^{2+}, plasma membrane blebbing appears not to depend upon this. However, it is still likely that both phenomena are due to thiol group oxidation, as indicated by their reversibility by DTT. It is possible that membrane blebbing is caused by the direct oxidation of thiol groups in cytoskeletal elements, thus leading to their disruption, and evidence that this can occur has recently been obtained [31].

Quin 2 appears to be equally effective in protecting cells previously exposed to paracetamol, as during co-incubation with the hepatotoxin. However, it is noteworthy that protection does occur during phase II incubation. This indicates that cytotoxicity requires the sustained elevation of cytosolic Ca^{2+} and that reduction of the Ca^{2+} levels at any time up until viability starts to deteriorate will serve to protect the cells. This is supported by the similarity in the time course for the effectiveness of DTT. Presumably, this prevents loss of viability by reducing disulphide groups in Ca^{2+}-translocases, which enables Ca^{2+} homeostasis to be restored.

It is believed that the mechanism of cell death in hepatocytes exposed to cytotoxic compounds may be the same as that which occurs during necrosis *in vivo*. The specific chelation of free cytosolic Ca^{2+} by Quin 2 in hepatocytes exposed to paracetamol prevents loss of viability, demonstrating a causative role of a sustained elevation of cytosolic Ca^{2+} in the events leading ultimately to cell death caused by this compound. It has also recently been shown that the chelation of intracellular Ca^{2+} prevents apoptotic cell death in immature thymocytes [32]. The events initiated by a sustained relase of cytosolic Ca^{2+} that lead to the death of the cell, and the extent to which these are the same in apoptosis and necrosis, have yet to be determined.

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