Cytoprotection by iloprost against paracetamol-induced toxicity in hamster isolated hepatocytes

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1 The ability of iloprost (ZK36374) to protect hamster isolated hepatocytes from the toxic effects of paracetamol and its reactive metabolite N-acetyl-*p*-benzoquinoneimine (NABQI) was investigated. The cytoprotection provided by iloprost was compared with that of N-acetyl-L-cysteine.

2 Treatment of hepatocytes with either NABQI (0.4 mM) or paracetamol (2 mM) alone resulted in a considerable loss of cell viability, as assessed by trypan blue exclusion or leakage of lactate dehydrogenase, accompanied by an increase in the percentage of viable cells that were blebbed. N-acetyl-L-cysteine (1.25 mM) pretreatment diminished the loss of cell viability and the percentage of blebbed cells resulting from exposure to NABQI or paracetamol, whereas iloprost (10^{-16} M to 10^{-10} M) pretreatment reduced only the loss of cell viability, not the percentage of viable cells exhibiting blebbing. Pretreatment with N-acetyl-L-cysteine significantly attenuated the depletion by paracetamol of glutathione and decreased the covalent binding of [¹⁴C]-paracetamol to cellular proteins, whereas iloprost was without any such effects.

3 The effects of iloprost and N-acetyl-L-cysteine were also investigated by use of a model of paracetamol toxicity in which it is possible to study the biochemical events leading to cell injury separate from the generation of toxic metabolites. Hamster hepatocytes were incubated with paracetamol (4 mM) for 90 min at 37°C during which metabolism of paracetamol occurs with minimal loss of cell viability. Following washing of cells, to remove paracetamol and its metabolites, there was a progressive loss of viability and increase in the percentage of cells exhibiting blebbing when incubated in buffer alone. Addition of either N-acetyl-L-cysteine (1.25 mM) or iloprost (10^{-14} M to 10^{-8} M), following washing, significantly reduced the expected loss of cell viability. Iloprost at concentrations outside this range was without effect.

4 Paracetamol toxicity to isolated hepatocytes could be prevented or delayed by treatment with either N-acetyl-L-cysteine or iloprost, but whereas the former prevented or even reversed plasma membrane blebbing with a resultant reduction in the percentage of viable cells that were blebbed, the prostanoid appeared only to delay the progression from plasma membrane blebbing to loss of viability. Hence, the percentage of viable cells that were ultimately blebbed following exposure to paracetamol was not significantly reduced by addition of iloprost.

5 Aspirin or ibuprofen exacerbated the loss of viability induced by prior incubation with paracetamol. Thus, there may be a role for endogenous prostaglandins in protecting hepatocytes from paracetamol toxicity.

6 Iloprost is cytoprotective without any effect upon toxin metabolism or detoxication. The mechanism of action of iloprost probably does not involve induction of prostaglandin synthesis or activation of the previously-characterized prostacyclin receptor.

Keywords: Cytoprotection; iloprost; hepatocytes; paracetamol; prostaglandins; toxicity

Introduction

The administration of exogenous prostaglandins to rats can prevent necrosis of the gastric mucosa following its exposure to a variety of noxious stimuli (Robert *et al.*, 1968), including mineral acids, alkali, boiling water, ethanol, corticosteroids, nonsteroidal anti-inflammatory compounds and bile acids (Robert, 1976; 1979; Lancaster & Robert, 1978; Robert *et al.*, 1979). This property of prostaglandins has been termed cytoprotection (Chaudhury & Jacobson, 1978), although more properly it should be described as organoprotection (Szabo & Szelenyi, 1987). It is now known that the cytoprotective effects of prostaglandins extend to tissues other than the stomach, including the heart (Lefer *et al.*, 1978; Ferrari *et al.*, 1989), pancreas (Manabe & Steer, 1980), kidney (Papanicolaou *et al.*, 1975; Ruwart *et al.*, 1981) and liver (Stachura *et al.*, 1980). It is likely that in many of these tissues cytoprotection is by a different, or additional mechanism, from that which operates in the stomach.

A number of compounds can cause hepatotoxicity, involving a variety of different mechanisms (Boobis *et al.*, 1989). Prostaglandins have been reported to prevent the hepatic

damage caused by many such agents, including: aflatoxin B₁ (Rush *et al.*, 1989), bromobenzene (Funck-Brentano *et al.*, 1984; Bursch & Schulte-Hermann, 1987), carbon tetrachloride (Ruwart *et al.*, 1981; Stachura *et al.*, 1981; Ujhelyi *et al.*, 1984; Guarner *et al.*, 1985; Bursch & Schulte-Hermann, 1987; Bursch *et al.*, 1989; Mihás *et al.*, 1991) and paracetamol (Stachura *et al.*, 1981; Guarner *et al.*, 1988). Hepatic cytoprotection by prostaglandins is apparent not only *in vivo* (Araki & Lefer, 1980; Stachura *et al.*, 1981; Funck-Brentano *et al.*, 1984; Bursch *et al.*, 1989; Ferrari *et al.*, 1989) but also in isolated hepatocytes (Ujhelyi *et al.*, 1984; Guarner *et al.*, 1985; Bursch *et al.*, 1989). This effect in isolated cells shows that prostaglandins can be directly 'cyto'-protective as opposed to 'organo'-protective (Szabo & Szelenyi, 1987), at least in the liver. Thus, at least one mode of action must be a direct effect on cells and not through secondary effects; such as on blood flow. Bursch and colleagues (Bursch & Schulte-Hermann, 1986; 1987; Bursch *et al.*, 1989) have reported that iloprost (ZK36374) can protect rat hepatocytes against the damage induced by carbon tetrachloride and bromobenzene. Iloprost ($0.1 \mu\text{g kg}^{-1} \text{ min}^{-1}$ *in vivo* and 6.9×10^{-9} M to 6.9×10^{-15} M in primary hepatocyte cultures) largely preserved normal hepatocellular morphology after intoxication (Bursch & Schulte-Hermann, 1986; 1987). The finding by these authors that iloprost protects cultured rat hepatocytes at concentrations as low as 6.9×10^{-15} M and that the efficacy of the pros-

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tanoid decreased at concentrations above 6.9×10^{-7} M suggests that iloprost is cytoprotective by means other than activation of the previously-characterized prostacyclin receptor. From the data of Bursch and colleagues (Bursch & Schulte-Hermann, 1986; 1987; Bursch *et al.*, 1989) it is not possible to exclude an action of iloprost on toxin metabolism, as protection against bromobenzene or carbon tetrachloride intoxication was obtained following their co-incubation with iloprost.

We have developed a well-defined model of cytotoxicity in which it is possible to study the biochemical events leading to cell injury separate from the generation of toxic metabolites (Tee *et al.*, 1986). In the present study, the effects of iloprost on the toxicity of paracetamol and its reactive metabolite (N-acetyl-*p*-benzoquinoneimine: NABQI) subsequent to the initiation of biochemical events that can lead to cell death have been investigated. Cytoprotection by prostanooids has also been compared with the protection afforded by N-acetyl-L-cysteine, the mechanism of which is relatively well understood.

Methods

Isolation of hepatocytes

Hepatocytes were isolated from male Golden Syrian hamsters (90–110 g) by the collagenase perfusion technique previously described (Tee *et al.*, 1986). However, the calcium and magnesium-free Earle's balanced salt solution (EBSS)-based perfusion media did not contain gentamicin sulphate.

The number of viable cells in the final suspension was determined by counting an aliquot of the suspension in an improved Neubauer counting chamber viewed under phase-contrast illumination. The viability of the cells was assessed by their ability to exclude trypan blue (0.05% w/v in the final volume). The cell suspension was adjusted to 2×10^6 viable cells ml^{-1} and was incubated at 37°C for 30 min with gentle shaking at 25 cycles min^{-1} . The cells were then sedimented at 100 *g* for 1 min and the supernatant fraction, containing dead and damaged cells, was discarded. The cells were resuspended in incubation medium (EBSS containing 8 mM sodium bicarbonate, 10 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 12 mM sodium phosphate, 1.05 mM calcium chloride, 0.73 iu ml^{-1} insulin (from bovine pancreas) and 0.1 mM hydrocortisone-21-hemisuccinate) at 4°C before use. This 30 min preincubation aided in the isolation of a cell population of high viability showing minimal blebbing (initial viability $97.6 \pm 1.6\%$ $n = 18$).

N-acetyl-*p*-benzoquinoneimine (NABQI)-synthesis and quantification

NABQI was synthesized by the oxidation of paracetamol (Aldrich Chemical Company, Gillingham, Dorset) in chloroform (Aristar grade: BDH Limited, Poole, Dorset) with freshly prepared silver oxide (Huggett & Blair, 1983). The solution of NABQI in chloroform was stored under liquid nitrogen until required. NABQI concentrations were measured directly by straight phase high performance liquid chromatography (h.p.l.c.) (Huggett & Blair, 1983) using known standards.

Assay of 'total' intracellular glutathione content

The intracellular glutathione content, comprising reduced glutathione (GSH) and glutathione disulphide (GSSG), was determined by the method of Akerboom & Sies (1981). Intracellular glutathione content has been expressed as nmol GSH per 10^6 cells.

Covalent binding of metabolites of [^{14}C]-paracetamol to proteins of isolated hepatocytes

Ring-labelled [^{14}C]-paracetamol (specific activity: 19.5 mCi mmol^{-1}) was obtained from Sigma (Poole, Dorset), with a radiochemical purity of greater than 99%. The covalent binding of the metabolites of [^{14}C]-paracetamol to

hepatocyte proteins was determined by the method described previously (Tee *et al.*, 1986). Determination of protein concentration was by the method of Lowry *et al.* (1951). Covalent binding is expressed in nmol of paracetamol equivalents per milligram of protein.

Experimental protocols

Three different experimental protocols for paracetamol toxicity to isolated hepatocytes were employed in these studies; designated protocols A, B, C. In the first two, hepatocytes were preincubated with either iloprost or N-acetyl-L-cysteine, followed by incubation with either NABQI (protocol A), or paracetamol (protocol B). In protocol A, 10 ml of cell suspension (2×10^6 cells ml^{-1}) were preincubated for 30 min with iloprost (10^{-18} M to 10^{-6} M) or with N-acetyl-L-cysteine (1.25 mM). The cells were then incubated with 0.4 mM NABQI, in the continuing presence of the iloprost or N-acetyl-L-cysteine, for 30 min. Aliquots of the cell suspension were removed at various times for assessment of cell viability. In protocol B, 10 ml samples of cell suspension (2×10^6 cells ml^{-1}) were preincubated for 30 min with iloprost or N-acetyl-L-cysteine. The cells were then incubated for 3 h with 2 mM paracetamol in the continuing presence of the iloprost or N-acetyl-L-cysteine. Aliquots of cell suspension were removed at 1, 2 and 3 h for viability assessment. Protocol C was adapted from the two-phase model used by Tee *et al.* (1986) for the study of antidotes to paracetamol toxicity. Ten ml of hepatocytes (2×10^6 cells ml^{-1}) were incubated with 4 mM paracetamol for 90 min (phase 1). The cells were then washed free of paracetamol and metabolites, including NABQI (Tee *et al.*, 1986; 1987), by centrifuging the cells at 100 *g* for 1 min and replacement of the supernatant with fresh incubation medium. This washing of the cells was repeated three times. The cells, in a final volume of 10 ml incubation medium, were then incubated with iloprost (10^{-18} M to 10^{-6} M) or N-acetyl-L-cysteine (1.25 mM) for a further 4.5 h (phase 2). Aliquots of cell suspension were removed at intervals for the assessment of cell viability. Controls contained an appropriate volume of the respective vehicle. Iloprost (ZK36374), in a vehicle buffered to pH 8.3 (1.2 mg Tris base, 8.9 mg sodium chloride, $10 \mu\text{l}$ of 96% ethanol brought to pH 8.3 with 0.1 N hydrochloric acid and made up to 1 ml with distilled water) was a kind gift of Schering A. G., Berlin. Iloprost was diluted to suitable stock concentrations (10^{-16} M to 10^{-4} M) by serial 1 in 10 dilutions in phosphate buffered saline (PBS), pH 7.4. N-acetyl-L-cysteine was obtained from Sigma (Poole, Dorset) and was dissolved in PBS before use. All cell incubations were carried out at 37°C with gentle shaking at 35 cycles min^{-1} . Hepatocyte suspensions were saturated with 95% O_2 : 5% CO_2 every 60 min during incubation.

Assessment of viability

The viability of cells was assessed by trypan blue exclusion or lactate dehydrogenase (LDH) leakage as described previously (Tee *et al.*, 1985). Evaluation of the extent of plasma membrane blebbing was performed in conjunction with the trypan blue exclusion assay. Cells excluding trypan blue which exhibited blebbing were counted. The percentage of blebbed cells was calculated as follows:

$$\%B = \frac{T_b}{T_{be}} \times 100$$

where %B = percentage of blebbed cells, T_b = total number of cells excluding trypan blue which were blebbed and T_{be} = total number of cells which excluded trypan blue.

The synthesis of prostaglandins PGE_2 , $\text{PGF}_{2\alpha}$ and PGI_2 by hepatocytes

Hamster isolated hepatocytes in 1 ml of incubation medium were incubated with one of the following for 30 min at 37°C : incubation medium; 10^{-4} M aspirin; 10^{-4} M ibuprofen;

10^{-14} M iloprost or 2 mM paracetamol. After incubation, cells were centrifuged at 1000 *g* for 2 min and the supernatant was removed. Internal standard (2 ng), a mixture of the deuterated prostaglandins, PGE₂, PGF_{2 α} and 6-oxo-PGF_{1 α} , was added to the supernatant. Mixed deuterated prostaglandin standards and non-deuterated standards were a kind gift from Upjohn Company, Michigan, U.S.A. A standard curve for each prostaglandin was constructed by preparing a range of deuterated and non-deuterated standards from 0 to 2000 pg ml⁻¹. Standard curves were constructed by plotting the peak area ratio of the non-deuterated prostaglandin to that of the corresponding deuterated prostaglandin against known concentration. Samples containing internal standard were acidified and extracted with Sep Pak C18 cartridges (Millipore Ltd., Watford). Following washing of the cartridge with 10 ml distilled water the prostaglandins were eluted with 7 ml ethyl acetate. After evaporation to dryness under nitrogen, samples were transferred to half-dram glass vials in 0.5 ml methanol which was removed under a nitrogen stream.

Samples were converted to their methoxime-trimethylsilyl ether-bistrifluoromethylbenzylester derivatives and analysed by capillary column gas chromatography-electron capture mass spectrometry (Barrow & Taylor, 1987). Chromatography was carried out on a CPSil 5 GC column (Chrompak, London) with helium as carrier gas. The column temperature was programmed to increase at 20°C min⁻¹ from 200°C to 325°C.

To investigate the effect of inhibitors of prostaglandin synthesis on paracetamol toxicity, hepatocytes were incubated with 2 mM paracetamol for 45 min (phase 1) and, following washing, were incubated with one of the following agents during phase 2: incubation medium; 10^{-4} M aspirin; 10^{-4} M ibuprofen or 10^{-14} M iloprost.

Statistical analysis

The data are presented as means $\pm$ s.e. of *n* experiments; each one utilising cells derived from a different hamster. Differences between mean values were tested for statistical significance by one- and two-way analysis of variance. Differences between individual treatment groups were tested by Scheffé's method (Scheffé, 1957), which corrects for multiple comparisons (Armitage & Berry, 1987). The null hypothesis was rejected at $P \leq 0.05$.

Results

Pretreatment of hepatocytes with iloprost or N-acetyl-L-cysteine before exposure to NABQI: protocol A

Incubation with any of the respective vehicles was without effect on hepatocyte viability. In the absence of NABQI, treatment of hepatocytes with iloprost alone, at concentrations of up to 10^{-8} M, had no effect on either their viability or degree of plasma membrane blebbing (data not shown). Incubation of cells with NABQI (0.4 mM) alone caused a considerable decrease in total cell viability, to $26.8 \pm 3.2\%$ after 30 min of exposure (Figure 1a). Iloprost, at concentrations in the range of 10^{-16} M to 10^{-10} M, significantly reduced the loss of viability induced by NABQI (Figure 1a). Neither 10^{-18} M nor 10^{-8} M iloprost had any significant effect on the loss of viability caused by NABQI (Figure 1a). Pretreatment of cells with N-acetyl-L-cysteine (1.25 mM) for 30 min before exposure to NABQI, significantly ($P < 0.001$) reduced the loss of cell viability (Figure 1a). Following exposure to NABQI (0.4 mM) for 30 min, the remaining viable hepatocytes exhibited a modest degree of plasma membrane blebbing ($14.9 \pm 3.6\%$) (Figure 1b). Preincubation of the cells with N-acetyl-L-cysteine (1.25 mM) significantly ($P < 0.005$) reduced the percentage of viable cells which were blebbed after exposure to NABQI (Figure 1b), whilst preincubation with iloprost (10^{-18} M to 10^{-8} M) had no significant effect on the percentage of viable cells exhibiting blebbing (Figure 1b).

Figure 1 The effect of preincubation of hepatocytes with iloprost (10^{-18} M to 10^{-8} M) or N-acetyl-L-cysteine (1.25 mM) on the toxicity of N-acetyl-p-benzoquinoneimine (NABQI) (protocol A): (a) the percentage of viable cells at 30 min assessed by trypan blue exclusion; (b) the percentage of viable cells which were blebbed. All but the control cells (open column, 9) were treated with NABQI (0.4 mM). The treatments were as follows: column (1) NABQI alone; columns (2)–(7) NABQI plus iloprost: 10^{-18} M (2); 10^{-16} M (3); 10^{-14} M (4); 10^{-12} M (5); 10^{-10} M (6); 10^{-8} M (7); column (8) NABQI and N-acetyl-L-cysteine (1.25 mM); column (9) control. Results are expressed as the mean with s.e. shown by vertical bars (*n* = 9). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffé's method. The null hypothesis was rejected at $P \leq 0.05$. *** $P < 0.001$; ** $P < 0.005$, compared with cells treated with NABQI only.

Pretreatment of hepatocytes with iloprost or N-acetyl-L-cysteine before exposure to paracetamol: protocol B

Incubation of hepatocytes with paracetamol (2 mM) alone caused a progressive deterioration of cell viability with time, as assessed by trypan blue exclusion (Figure 2a) and LDH leakage (data not shown), and an increase in the degree of cell blebbing (Figure 2b). Iloprost pretreatment of cells significantly reduced the loss of viability caused by incubation with paracetamol. Iloprost had no significant effect on the percentage of viable cells exhibiting blebbing following paracetamol exposure (Figure 2b). Pretreatment of cells for 30 min with N-acetyl-L-cysteine (1.25 mM) before exposure to paracetamol (2 mM) significantly reduced the loss of viability (Figure 2a) and substantially reduced the percentage of viable cells exhibiting plasma membrane blebbing (Figure 2b), compared with cells treated with paracetamol alone.

The glutathione content of control cells was initially 35.8 ± 9.8 nmol per 10^6 hepatocytes and at the end of the experiment it was 32.6 ± 5.9 nmol per 10^6 hepatocytes. The glutathione levels of treated cells are expressed as a percentage

Figure 2 The effect of pretreatment of hepatocytes with iloprost (10^{-14} M) or N-acetyl-L-cysteine (1.25 mM) on the toxicity of paracetamol (protocol B): (a) viability as a percentage of that of untreated cells assessed by trypan blue exclusion; (b) the percentage of viable cells which were blebbed. Cells were treated as follows: paracetamol (2 mM) alone (▲); paracetamol (2 mM) following pretreatment with iloprost (10^{-14} M) (■) and paracetamol (2 mM) following pretreatment with N-acetyl-L-cysteine (1.25 mM) (●). Results are expressed as the mean with s.e. shown by vertical bars ($n = 9$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffé's method. The null hypothesis was rejected at $P \leq 0.05$. *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only.

of that of control cells. Exposure of hepatocytes to paracetamol resulted in a marked depletion of cellular glutathione to less than 25% of control values (Figure 3). Iloprost alone had no effect on the glutathione content of hepatocytes, at any concentration (Figure 3). Pretreatment with N-acetyl-L-cysteine (protocol B) significantly attenuated the effects of paracetamol on glutathione levels ($P < 0.05$ at 3 h) (Figure 3). Iloprost, at concentrations that were cytoprotective (10^{-14} M or 10^{-12} M), did not prevent the depletion of glutathione caused by incubation with paracetamol (Figure 3).

Following exposure of hepatocytes to [14 C]-paracetamol (2 mM) for 3 h there was 0.62 ± 0.03 nmol paracetamol equivalent covalently bound per mg of hepatic protein (Table 1). Pretreatment with N-acetyl-L-cysteine (protocol B) significantly ($P < 0.001$) decreased the covalent binding of [14 C]-paracetamol to cellular proteins (Table 1). In contrast, pretreatment of cells with 10^{-14} M, 10^{-12} M or 10^{-9} M iloprost had no significant effect on the extent of covalent binding of [14 C]-paracetamol to cellular proteins (Table 1).

Two-phase model of paracetamol toxicity: protocol C

Hamster hepatocytes incubated in incubation medium alone using protocol C, without exposure to paracetamol, main-

Figure 3 The effect of pretreating hepatocytes with iloprost (10^{-14} M or 10^{-12} M) or N-acetyl-L-cysteine (1.25 mM) followed by paracetamol (protocol B) on the GSH content as a percentage of that of control (untreated) hepatocytes after 3 h of incubation. Hepatocytes were treated with buffer alone, iloprost or N-acetyl-L-cysteine for 30 min prior to incubation in buffer with or without paracetamol. The cells were treated as follows: column (1) paracetamol (2 mM) alone; (2) paracetamol (2 mM) following pretreatment with N-acetyl-L-cysteine (1.25 mM); (3) iloprost (10^{-14} M) alone; (4) paracetamol (2 mM) following pretreatment with iloprost (10^{-14} M) alone; (5) iloprost (10^{-12} M) alone; (6) paracetamol (2 mM) following pretreatment with iloprost (10^{-12} M) alone. Results are expressed as the mean with s.e. shown by vertical bars ($n = 9$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffé's method. The null hypothesis was rejected at $P \leq 0.05$. ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only.

tained their viability well for 6 h (initial viability: $88.8 \pm 2.6\%$; final viability: $68.2 \pm 2.4\%$; $n = 6$; as assessed by trypan blue exclusion). There was a close correlation between viability assessed by LDH leakage and by trypan blue exclusion (Figures 4a,b). Treatment with iloprost alone, at concentrations of up to 10^{-8} M, or with N-acetyl-L-cysteine (1.25 mM) had minimal effects on cell viability (data not shown). After incubation in incubation medium alone for 6 h using protocol C the percentage of viable cells exhibiting plasma membrane blebbing increased slightly, from $14.4 \pm 1.5\%$ to $18.7 \pm 1.2\%$.

Table 1 Effect of iloprost or N-acetyl-L-cysteine in protocol B on the covalent binding of [14 C]-paracetamol to cellular proteins of hepatocytes

	Covalent binding of [14 C]-paracetamol (nmol mg ⁻¹ protein)
Paracetamol only	0.62 ± 0.03
Pretreatment with iloprost (10^{-14} M) before exposure to paracetamol	0.49 ± 0.13
Pretreatment with iloprost (10^{-12} M) before exposure to paracetamol	0.56 ± 0.07
Pretreatment with iloprost (10^{-9} M) before exposure to paracetamol	0.52 ± 0.09
Pretreatment with N-acetyl-L-cysteine (1.25 mM) before exposure to paracetamol	0.34 ± 0.01***

Hepatocytes were pretreated with iloprost or N-acetyl-L-cysteine and then incubated with [14 C]-paracetamol (2 mM) for 3 h. Covalent binding (nmol mg⁻¹ protein) in pretreated samples was compared with that in cells which were treated with paracetamol only. Results have been expressed as the mean $\pm$ s.e. ($n = 6$). *** $P < 0.001$ compared with cells treated with paracetamol only, Student's paired *t* test.

Figure 4 Effects of iloprost in a two-phase model of paracetamol toxicity: protocol C. (a) Viability as a percentage of that of untreated cells, as assessed by lactate dehydrogenase leakage. The cells were treated as follows: paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer alone during phase 2 ($\blacktriangle$); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing N-acetyl-L-cysteine (1.25 mM) during phase 2 ($\blacksquare$); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing iloprost (10^{-10} M) during phase 2 ($\bullet$). Results are expressed as the mean with s.e. shown by vertical bars ($n = 6$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leq 0.05$. There was a significant difference between treatment with paracetamol alone and treatment with paracetamol followed by either iloprost or N-acetyl-L-cysteine.

Exposure of cells to either iloprost (10^{-8} M) or N-acetyl-L-cysteine (1.25 mM) during phase 2 had no significant effect on the percentage of viable cells that were blebbed.

Cells treated with paracetamol (4 mM) during phase 1 maintained their viability throughout this period (Figure 4a), although there was an increase in the percentage of viable cells that were blebbed, beyond that seen in untreated cells (Figure 4c). Following washing of the cells, to remove the paracetamol and its metabolites, there was a progressive loss in cell viability during incubation in incubation medium alone (Figure 4a). The percentage of cells showing blebbing increased throughout the phase 2 incubation (Figure 4c). Treatment of such cells with N-acetyl-L-cysteine (1.25 mM) during phase 2 significantly reduced the loss of cell viability (Figures 4a,b) and the percentage of viable cells exhibiting blebbing (Figure 4c) resulting from prior exposure to paracetamol. Incubation with iloprost (10^{-14} M to 10^{-8} M) during phase 2 significantly reduced the loss of cell viability, assessed by either LDH leakage (Figure 4a) or trypan blue exclusion (Figure 4b), caused by paracetamol. Iloprost, however, at concentrations outside this range was without effect (Figure 4b). At no concentration of iloprost tested was there any change in the percentage of viable cells showing blebbing (Figure 4c).

Synthesis of prostaglandins by hamster isolated hepatocytes

The most abundant of the prostaglandins synthesized by hepatocytes was PGE_2 ($208 \pm 52 \text{ pg ml}^{-1}$) (Table 2). $\text{PGF}_{2\alpha}$ was present at $124 \pm 19 \text{ pg ml}^{-1}$ but there was negligible 6-oxo- $\text{PGF}_{1\alpha}$, a stable rearrangement product of PGI_2 . Both aspirin and ibuprofen markedly inhibited prostaglandin synthesis, reducing the levels of all of the prostaglandins to below the limit of detection ($< 50 \text{ pg ml}^{-1}$). Iloprost (10^{-14} M) did not affect prostaglandin levels but paracetamol stimulated PGI_2 production ($P < 0.001$).

*** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only. (b) Viability as a percentage of that of untreated cells, as assessed by trypan blue exclusion, at 360 min after the start of phase 1. The cells were treated as follows: column (1) iloprost (10^{-10} M) alone; column (2) paracetamol (4 mM) during phase 1 followed by extensive washing and incubation in buffer alone during phase 2; column (3) paracetamol (4 mM) during phase 1 followed by extensive washing and incubation in buffer containing iloprost (10^{-6} M) during phase 2; column (4) as column (3), but with iloprost (10^{-8} M); column (5) as column (3) but with iloprost (10^{-10} M); column (6) as column (3) but with iloprost (10^{-12} M); column (7) as column (3) but with iloprost (10^{-14} M); column (8) as column (3) but with iloprost (10^{-16} M). Results are expressed as the mean with s.e. shown by vertical bars ($n = 6$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leq 0.05$. *** $P < 0.001$; ** $P < 0.005$, compared with cells treated with paracetamol only. (c) The percentage of viable cells which were blebbed. The cells were treated as follows: paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer alone during phase 2 ($\blacktriangle$); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing N-acetyl-L-cysteine (1.25 mM) during phase 2 ($\bullet$); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing iloprost (10^{-10} M) during phase 2 ($\blacksquare$). Results have been expressed as the mean with s.e. shown by vertical bars ($n = 6$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leq 0.05$. There was a significant difference between treatment with paracetamol alone and treatment with paracetamol followed by either iloprost or N-acetyl-L-cysteine. *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only.

Table 2 Effect of various treatments on prostaglandin levels of hamster isolated hepatocytes

Treatment	Prostaglandin concentration (pg per 10 ⁶ cells)		
	PGE ₂	PGF _{2α}	6-oxo-PGF _{1α}
Control	208 ± 52	124 ± 19	< 50
Aspirin (10 ⁻⁴ M)	< 50***	< 50***	< 50
Ibuprofen (10 ⁻⁴ M)	< 50***	< 50***	< 50
Iloprost (10 ⁻¹⁴ M)	210 ± 82	102 ± 45	< 50
Paracetamol (2 mM)	265 ± 32	100 ± 65	150 ± 35***

Hepatocytes were incubated with incubation medium (Control) or other treatments for 30 min at 37°C. Results have been expressed as the mean ± s.e. (*n* = 8). *** *P* < 0.001 compared with control cells, Student's *t* test.

Effect of altering the rate of prostaglandin synthesis on the toxicity of paracetamol

Hepatocytes were incubated with paracetamol (2 mM) for 45 min during phase 1. During phase 2, there was a progressive decrease in the percentage of viable cells, to 32.3 ± 5.2% (*n* = 8) after 3 h (Table 3). Incubation during phase 2 with either aspirin (10⁻⁴ M) or ibuprofen (10⁻⁴ M), both of which completely inhibited the synthesis of prostaglandins, exacerbated the loss of viability induced by prior incubation with paracetamol. Simultaneous exposure to aspirin (10⁻⁴ M) and iloprost (10⁻¹⁴ M) during phase 2 provided less protection than iloprost alone (Table 3).

Discussion

There have been a number of reports that iloprost (ZK36374), a stable analogue of prostacyclin with similar anti-aggregatory and vasodilator effects (Schrör *et al.*, 1981), protects cells against the toxicity of a variety of agents. However, the mechanism of action of this effect and the duration over which iloprost is effective are not known. We have now investigated, using freshly isolated hepatocytes, the effects of iloprost on the toxicity of paracetamol and its reactive metabolite NABQI.

Iloprost, when present at the time of addition of toxin to the cells (protocols A and B), significantly reduces the cytotoxicity of both paracetamol and NABQI. In protocol B 2 mM paracetamol was used but, owing to variation in the sensitivity of different groups of hamsters to paracetamol toxicity, 4 mM paracetamol was employed in protocol C. With protocol

C it is possible to dissociate the metabolic phase of paracetamol toxicity, during which the compound is activated and initiates those events that ultimately lead to cell death, from a phase of progressive biochemical and morphological deterioration, which leads eventually to irreversible changes and ultimately to loss of viability. Iloprost, added after washing the cells, prevents the loss of viability of paracetamol-pretreated hepatocytes during phase 2 incubation. Iloprost is effective against NABQI, which is directly cytotoxic, and is protective against paracetamol under conditions (protocol C) in which it cannot affect metabolism. Iloprost also does not affect GSH levels, either in the absence or, more significantly, in the presence of paracetamol. Further, iloprost does not affect the covalent binding of paracetamol to cellular protein. Thus, iloprost reduces the loss of cell viability without affecting either the metabolism or detoxication of paracetamol. The reported effectiveness of iloprost against galactosamine-induced injury (Stachura *et al.*, 1981; Noda *et al.*, 1986) provides further evidence that at least one mechanism of action of prostanoids is independent of the metabolism of the toxin.

N-acetyl-L-cysteine protects against the toxicity of both paracetamol and NABQI when present at the start of the incubation. Most, if not all, of the protective effect of N-acetyl-L-cysteine against the toxicity of these compounds, when present simultaneously, is by serving as a precursor for GSH synthesis which prevents the escape of the toxic metabolite. In protocol C, N-acetyl-L-cysteine is still protective despite being added after the metabolic phase of toxicity. However, its effect still appears to be mediated via the synthesis of GSH, the levels of which are largely restored to control values following addition of the compound. It is likely that GSH thus formed acts as a thiol-reducing agent, reactivating thiol groups oxidized by NABQI (Bruno *et al.*, 1988).

Whereas N-acetyl-L-cysteine prevents, or even reverses, the plasma membrane blebbing caused by NABQI or paracetamol, iloprost appears only to delay, but not prevent or reverse, plasma membrane blebbing. As iloprost is cytoprotective during phase 2, without affecting the levels of GSH or reversing plasma membrane blebbing, this suggests that whilst it can arrest those events leading to cell death, there must be additional ones leading to plasma membrane blebbing. Both blebbing and loss of viability are probably initiated by a common biochemical event, most likely thiol-group oxidation, as thiol-reducing agents such as dithiothreitol and GSH, synthesized from N-acetyl-L-cysteine, can prevent both processes (Tee *et al.*, 1986). The mechanism of action of iloprost in providing cytoprotection without reversing plasma membrane blebbing is not known.

In agreement with the results of others (Bursch & Schulte-Hermann, 1987; Ferrari *et al.*, 1989) the cytoprotective effects of iloprost are apparent at very low doses of the prostanoid, as little as 10⁻¹⁴ M showing significant protection. Given Avogadro's number (6.02 × 10²³) this concentration corresponds to only 30 molecules per hepatocyte. The K_{act} for prostacyclin at its receptor, the receptor through which iloprost exerts its pharmacological effects, has not been determined for hepatocytes. However, for human platelets it is approximately 10⁻⁸ M (Lombroso *et al.*, 1984). Thus, it is unlikely that iloprost is cytoprotective through activation of this receptor.

The role of endogenous prostaglandins in protection against hepatic injury *in vitro* was investigated by Guarner *et al.* (1985). They demonstrated that inhibition of prostaglandin synthesis by indomethacin led to an increase in the cytotoxicity of carbon tetrachloride. Treatment of mice with OKY1581, an inhibitor of thromboxane synthetase, increased prostacyclin synthesis, and protected the animals against paracetamol toxicity (Guarner *et al.*, 1988). The enhanced toxicity seen in protocol C following exposure of cells to aspirin or ibuprofen supports a role for endogenous prostaglandins in protecting hepatocytes against cytotoxic insult.

Clearly, iloprost is a potent cytoprotective agent in isolated hepatocytes against the toxicity of paracetamol. Its mechanism of action is quite different from that of N-acetyl-L-cyste-

Table 3 Effect of inhibition of prostaglandin synthesis on the toxicity of paracetamol

Treatment	Percentage of cells which were viable
Control	88.0 ± 3.2%***
Paracetamol (2 mM)	32.3 ± 5.2%
Paracetamol (2 mM) + aspirin (10 ⁻⁴ M)	18.4 ± 3.1%**
Paracetamol (2 mM) + ibuprofen (10 ⁻⁴ M)	20.2 ± 4.6%*
Paracetamol (2 mM) + aspirin (10 ⁻⁴ M) + iloprost (10 ⁻¹⁴ M)	72.8 ± 5.2%***
Paracetamol (2 mM) + iloprost (10 ⁻¹⁴ M)	80.4 ± 3.9%***

Hepatocytes, in incubation medium, were incubated with paracetamol (2 mM) or incubation medium for 45 min. Following washing, cells were incubated with one or more of the following for a further 3 h: incubation medium; 10⁻⁴ M aspirin; 10⁻⁴ M ibuprofen or 10⁻¹⁴ M iloprost. Controls were incubated with incubation medium throughout both phases. After the 3 h incubation samples were taken for the assessment of viability by trypan blue exclusion. Results have been expressed as the mean ± s.e. (*n* = 8). *** *P* < 0.001; ** *P* < 0.005; * *P* < 0.01, compared with cells treated with paracetamol only, Student's *t* test.

ine. Iloprost does not have any effect on the metabolism or detoxication of paracetamol. The novel mechanism of action of iloprost in providing protection against paracetamol toxicity suggests events in the progression of cell injury to cell death may be open to pharmacological manipulation.

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