RESEARCH PAPER

α₁-Adrenoceptor antagonists prevent paracetamol-induced hepatotoxicity in mice

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Background and purpose: Paracetamol, a major cause of acute liver failure (ALF) represents a significant clinical problem. Adrenoceptor stimulation or antagonism can modulate chemical-induced hepatotoxicity. We investigated the role of endogenous catecholamines and α₁-adrenoceptors in the development of paracetamol- induced hepatotoxicity.

Experimental approach: Paracetamol ($3.5\,mmol\,kg^{-1}$) was administered to male CD-1 mice, with and without α₁-adrenoceptor antagonists (prazosin, doxazosin, terazosin and tamsulosin; $35.7\,\mu mol\,kg^{-1}$). Serum transaminases and hepatic glutathione (GSH) levels were assessed as markers of hepatic damage. Paracetamol bioactivation was assessed by covalent binding, hepatic and urinary conjugate formation and uridine glucuronosyltransferase activity. Plasma catecholamines levels and hepatic congestion were also analysed.

Key results: Plasma catecholamine levels were significantly elevated 5 h post paracetamol administration. Prazosin prevented hepatotoxicity when administered 1 h before a toxic paracetamol insult and importantly, when administered up to 1 h post paracetamol injection. Prazosin had no effect on paracetamol-induced depletion of hepatic GSH, paracetamol bioactivation or paracetamol-induced transcription of defence genes. Paracetamol toxicity is associated with marked accumulation of erythrocytes within hepatic sinusoids and prazosin completely prevented this accumulation.

Conclusion and implications: Paracetamol-induced hepatocellular damage is associated with increased circulating catecholamines. α₁-Adrenoceptor antagonists conferred complete protection from paracetamol -induced hepatotoxicity. Protection was associated with absence of hepatic erythrocyte accumulation. Increased catecholamine levels may contribute to the pathophysiology of paracetamol-induced hepatotoxicity by compromising hepatic perfusion. Protection against paracetamol toxicity by α₁ antagonists in mice has implications for therapeutic management of patients presenting with paracetamol overdose and ALF.

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Introduction

The widely used analgesic, paracetamol, is extremely safe at therapeutic doses. However, overdose or chronic use of high-dose paracetamol has been the major cause of hospital admissions and indication for emergency liver transplantation for acute liver failure (ALF) in the UK, over the last 30 years (Bernal, 2003). It also currently accounts for approximately 40% of cases of ALF in the USA (Lee, 2003). At therapeutic levels, paracetamol is primarily metabolized in the liver by glucuronidation and sulphation; however, a

small proportion undergoes cytochrome P450 (CYP450)-mediated bioactivation to *N*-acetyl-*p*-benzoquinoimine (NAPQI), which is rapidly quenched by glutathione (GSH) (Raucy *et al.*, 1989; Thummel *et al.*, 1993).

After an overdose of paracetamol, elevated levels of the toxic NAPQI metabolite are generated, which can extensively deplete hepatocellular GSH and covalently modify cellular proteins resulting in hepatocyte death. The precise biochemical mechanism of cell necrosis is not fully understood. However, it is generally recognized that there is simultaneous involvement of covalent binding, lipid peroxidation and oxidative stress, each contributing to hepatocellular damage (Jollow *et al.*, 1973; Mitchell *et al.*, 1973; Hinson, 1980; Bessems and Vermeulen, 2001). More recent work has demonstrated that liver damage is clearly a

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multi-cellular event, with clear evidence for a role of cells of the innate immune system in tissue damage (Liu and Kaplowitz, 2006).

Adrenergic modulation of chemical-induced hepatotoxicity has been recognized for several decades. Early studies performed by Calvert and Brody utilized the model hepatotoxin, carbon tetrachloride (CCl_4), and demonstrated the ability to diminish hepatotoxicity via adrenergic blockade and adrenalectomy (Brody and Calvert, 1960; Calvert and Brody, 1960; Brody et al., 1961). This led to the hypothesis that catecholamines played a role in CCl_4 toxicity. Further studies demonstrated the potentiation of toxicity following administration of catecholamines, adrenergic agonist co-administration, and electrical or physical stimulation of the nervous system possibly involving α_2-adrenoceptor stimulation (Larson and Plaa, 1965; Schwetz and Plaa, 1969; Iwai et al., 1986; Iwai and Shimazu, 1988; Roberts et al., 1991, 1994). The mechanism of potentiation of CCl_4 hepatotoxicity remains uncertain but does not involve modulation of hepatic GSH content (Roberts et al., 1997).

Kerger and colleagues demonstrated dramatic reductions in bromobenzene-induced hepatotoxicity with concurrent non-selective α-adrenoceptor antagonist administration (Kerger et al., 1988a, b, 1989). Yohimbine, the selective α_2-adrenoceptor antagonist was ineffective in providing hepatoprotection (Harbison et al., 1996) implying a role for α_1-adrenoceptors, which was later confirmed with prazosin (1-(4-amino-6, 7-dimethoxy-2-quinazolinyl)-4-(2-furoyl) piperazine hydrochloride), a selective α_1 antagonist (Harbison et al., 1996).

Previous studies have shown that the α-adrenoceptor agonists phenylpropanolamine (non-selective α) and phenylephrine (α_1) potentiate paracetamol-induced hepatotoxicity in mice (Harbison et al., 1991; James et al., 1993) when administered to coincide with maximal GSH depletion. On the basis of these previous observations, we hypothesized that endogenous catecholamine release is induced by toxic doses of paracetamol and that blockade of adrenoceptors will prevent paracetamol-induced hepatotoxicity. In this study, we have tested this hypothesis by measuring catecholamine levels following paracetamol administration and assessed the effect of α-adrenoceptor antagonism with selective α_1 antagonists.

Prazosin is a selective α_1-adrenoceptor antagonist clinically used to treat hypertension. The peripheral vasodilatory properties of prazosin are due to postsynaptic α blockade. The mode of action of prazosin is confined primarily to the arterioles and is associated with a reduction in peripheral vascular resistance and mean arterial pressure. Doxazosin and terazosin are also quinazoline antihypertensives, structurally related to prazosin with similar metabolic elimination pathways. Tamsulosin ((−)-(R)-5-[2-[[2-(oethoxyphenoxy)ethyl]amino]-propyl]-2-methoxybenzenesulphonamide hydrochloride) is a subtype-selective α_1 antagonist (α_{1A}) of a different chemical class used to improve urinary obstruction in benign prostatic hypertrophy (Soeishi et al., 1996). In these studies, we have investigated the role of endogenous catecholamines and α_1-adrenoceptors in the modulation of paracetamol-induced hepatotoxicity.

Materials and methods

Animal dosing regimen
Protocols were undertaken in accordance with criteria outlined in a licence (PPL no. 40/2544) granted under the UK Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. Animals were housed in a light controlled room at constant temperature and were supplied with food and water ad libitum.

Dosing regimen. Male CD-1 mice ($n = 4$–12, 25–35 g) were administered a single dose of paracetamol (1 or 3.5 mmol kg^{-1}; i.p.) in warmed saline or vehicle control. Animals were killed after 1, 5 or 24 h by CO_2 asphyxiation. Blood was collected via cardiac puncture and livers were removed immediately, rinsed in saline (0.9%) and snap frozen in liquid nitrogen. Tissues were stored at −80 °C until use.

Endogenous catecholamine analyses. Male CD-1 mice ($n = 4$) were administered a single dose of prazosin (35.7 μmol kg^{-1} in corn oil; i.p.) 1 h prior to a subtoxic dose of paracetamol (3.5 mmol kg^{-1} in saline, i.p.). Animals were monitored in a darkened, quiet room to minimize stress. Animals were individually killed by an overdose of pentobarbital (Sagatal). It is possible that the actual procedure of killing the animal may raise circulating catecholamine levels, which could then potentially mask any (paracetamol ± prazosin)-induced changes. However, pilot studies confirmed that this was not the case. Blood was collected immediately via cardiac puncture into lithium heparin tubes containing EGTA and GSH kept on ice. Plasma was separated via centrifugation at 780 g for 10 min. The blood from two animals was pooled for each sample to ensure sufficient sample volume. Samples were stored at −80 °C.

Hepatotoxicity protection experiments. Male CD-1 mice ($n = 4$–12; 25–35 g) were administered a single dose of prazosin, doxazosin, terazosin or tamsulosin (35.7 μmol kg^{-1} emulsion in corn oil; i.p.) 1 h prior to a toxic dose of paracetamol (3.5 mmol kg^{-1} in saline; i.p.). Mice were also administered a single dose of prazosin (35.7 μmol kg^{-1}; i.p.) 30 min and 1 h after a toxic dose of paracetamol (3.5 mmol kg^{-1}; i.p.). Mice also received multiple doses of prazosin (35.7 μmol kg^{-1} in corn oil; i.p.) administered as a 15 min pre-dose followed by four further doses at 90-min intervals, alongside a toxic dose of paracetamol (3.5 mmol kg^{-1}; i.p.) at the 0 h time point. Mice receiving vehicle alone were used as controls. Animals were killed after 1, 5 or 24 h by CO_2 asphyxiation. Blood was collected via cardiac puncture. Livers were immediately removed and rinsed in saline (0.9%). Half the large lobe was stored in 10% neutral buffered formalin and the remainder of the liver was divided into aliquots and snap frozen in liquid nitrogen. Tissues were stored at −80 °C until use.

Metabolism analyses. Male CD-1 mice were maintained under anaesthesia with pentobarbital (70 mg kg^{-1}, i.p. in saline). The external jugular vein was cannulated (0.40 mm

ID, 0.8 mm OD fine bore polythene tubing, Portex Ltd, Kent, UK) to aid dosing. Animals received prazosin (35.7 μmol kg^{-1}, i.p.), 1 h prior to the bolus administration (over 2 min) of paracetamol (1.5 or 3.5 mmol kg^{-1}). Following CO_2 asphyxiation, livers were removed for immediate metabolite extraction.

Irreversible binding analyses. Male CD-1 mice ($n = 10$) were given paracetamol (3.5 mmol kg^{-1} in saline, i.p.) containing 10 μCi [^{3}H] paracetamol per mouse (stock 1.2 μCi μl^{-1}) in combination, in the presence or absence of pretreatment (1 h before paracetamol) with prazosin (35.7 μmol kg^{-1} in corn oil; i.p.). Livers were collected 5 h after paracetamol and stored at $-80\,^\circ$C.

Determination of serum alanine transaminase levels as a marker of hepatotoxicity

Serum was separated, via pulse centrifugation from blood that had been allowed to clot overnight. Serum alanine transaminase (ALT) levels were determined kinetically at 37 $^\circ$C using ThermoTrace Infinity ALT Liquid stable reagent, according to the manufacturer's instructions. ALT levels greater than 200 U l^{-1} that were statistically significant from controls were considered hepatotoxic (Kitteringham *et al.*, 2000; Goldring *et al.*, 2004).

Histopathological examination of H&E-stained hepatic sections

Following hepatic excision, a 4-mm transverse section was taken from the formalin-fixed tissue samples. The samples were dehydrated, and embedded in paraffin prior to 4-μm sections being cut. Sections were stained with haematoxylin and eosin (H&E) for evaluation. All histological examinations were performed blind by a single pathologist evaluating one liver section per animal. Liver sections were scored according to pre-defined criteria, where 0 is normal histology and 4 represents massive hepatocellular necrosis involving entire lobules

Analysis of plasma catecholamines as an indicator of the murine stress response following paracetamol administration

Plasma samples were organically extracted and analysed using HPLC with electrochemical detection according to the method of Forster and Macdonald (1999).

Assessment of hepatic GSH levels following paracetamol administration

Hepatic GSH levels were determined using a 5,5′-dithiobis(2-nitrobenzoic acid)—GSH disulphide recycling assay according to the method of Vandeputte *et al.* (1994).

Determination of hepatic paracetamol–Glutathione metabolite levels

Paracetamol-SG conjugate levels in liver extracts were quantified by HPLC as described previously (Elsby *et al.*, 2003) using authentic standards.

Determination of irreversible binding of radiolabelled paracetamol to hepatic proteins as a marker of reactive metabolite formation

Radiolabelled material irreversibly bound to precipitated protein was measured after removal of unbound drug by exhaustive solvent extraction according to the method of Pirmohamed *et al.* (1995). Irreversible binding of radio-labelled paracetamol is expressed as percentage bound per milligram hepatic protein.

Assessment of hepatic defence gene response via reverse transcriptase-PCR of GCLC and HO-1 mRNA

Total cellular hepatic RNA was extracted using the method of Chomczynski and Sacchi (1987). Briefly, liver (100 mg) was homogenized in ultraPURE TRIzol reagent according to the manufacturer's instructions. The purity, integrity and concentration of isolated total RNA was quantified by agarose gel electrophoresis and spectrophotometry, respectively. cDNA was synthesized using the Promega reverse transcription system according to the manufacturer's instructions using 1 μg total RNA as a template.

Glutamate cysteine ligase catalytic subunit (GCLC) was co-amplified with the constitutively expressed housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) according to the method of Kitteringham *et al.* (2000). The following primer pairs were used:
GCLC, Forward 5′-GCAATGCATCCTGCACCACC-3′,
Reverse 5′-GGTCTGGGATGGAAATTGTG-3′GAPDH,
Forward 5′-ATCCTCCAGTTCCTGCACAT-3′,
Reverse 5′-TGTGAATCCAGGGCAGCCTA-3′.

Optimization of reverse transcriptase-PCR assay conditions were performed on cDNA isolated from control and treated mice. Primer concentrations, volume of cDNA and PCR cycle number were optimized during linearity studies. Reactions contained cDNA (5 μl of a 1:10 dilution in nuclease-free water) combined with 4 μM each of the GCLC and GAPDH forward and reverse primers, 50 μM of each dNTP, 1.25 U *Taq* DNA polymerase and 5 μl of PCR buffer (100 mM Tris-HCl (pH 8.8); 500 mM KCl, 0.8% Nonidet P40) in a total volume of 50 μl. Amplification was initiated for 3 min of denaturation at 94 $^\circ$C followed by 35 cycles of 30 s at 94 $^\circ$C (denaturation), 1 min at 53 $^\circ$C (primer annealing) and 1 min at 72 $^\circ$C (polymerization) with a final elongation step of 72 $^\circ$C for 10 min. The GCLC and *GAPDH* PCR products were 390 and 670 bp, respectively.

Haem-oxygenase 1 (HO-1) was co-amplified with *cyclophilin* using a method designed in our laboratory. The following primer pairs were used:
HO-1, Forward 5′-GCTGAGTTCATGAAGAACTT-3′,
Reverse 5′-AGACGATTTACATAGTGCT-3′;Cyclophilin,
Forward 5′-GTCTGCTTCGAGCTGTTTGCA-3′,
Reverse 5′-GGGTGCTCTCCTGAGCTACAG-3′.

Reactions contained 0.04 μM HO-1 forward and reverse primers along with 0.08 μM forward and reverse cyclophilin primers, 50 μM of each dNTP, 0.4 U *Taq* DNA polymerase and 5 μl of PCR buffer in a total volume of 50 μl. Amplification was initiated for 5 min of denaturation at 94 $^\circ$C followed by 35 cycles of 10 s at 94 $^\circ$C (denaturation), 30 s at 48 $^\circ$C (primer annealing) and 30 s at 72 $^\circ$C with a final elongation step of

$72\,^{\circ}C$ for 5 min. The HO-1 and cyclophilin PCR products were 261 and 450 bp, respectively.

The GAPDH and cyclophilin housekeeping genes were not altered by either paracetamol or prazosin treatments and were therefore used to normalize hepatic defence gene expression data.

Quantitation of hepatic congestion using CFSE-labelled erythrocytes

Erythrocytes were purified from heparinized blood, obtained from donor mice, by density centrifugation over Lympho-prep. Erythrocytes were washed extensively, suspended in Hank's Buffered Salt Solution (HBSS) and fluorescently labelled with $10\,\mu M$ of either carboxyfluorescein diacetate succinimidyl ester (CFSE) or tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) at $37\,^{\circ}C$ for 20 min. Labelling was quenched with 10% fetal bovine serum, cells were washed and CFSE-labelled cells were intravenously injected into the tail vein of CD-1 mice, which were left overnight before administration of test compounds. Animals were killed by CO_2 asphyxiation 5 h after paracetamol ± prazosin treatment. Blood was collected via cardiac puncture, livers were removed, rinsed briefly in saline and dispersed through a wire mesh strainer with heparinized HBSS to release trapped erythrocytes. Cells were passed through a 40-μm cell strainer to remove tissue debris. Erythrocytes were separated from the filtrate using a Lymphoprep density gradient centrifuga-tion. The erythrocyte pellet was resuspended in 1 ml HBSS to which a fixed quantity of TRITC-labelled erythrocytes were added. These served as an internal control to assist quantitative analysis. Hepatic congestion was quantified using bivariate flow cytometry analysis (Coulter Epics, XL software; Beckman Coulter, High Wycombe, Buckingham-shire, UK) of CFSE-labelled erythrocytes recovered from the livers of treated and control animals. Erythrocytes were electronically gated based on their scatter profiles and the fluorescently labelled erythrocytes were detected and counted. CFSE erythrocytes and TRITC erythrocytes fluor-esce at different wavelengths and therefore can be distin-guished on the flow cytometer. CFSE-labelled erythrocytes were counted until 4000 TRITC-labelled erythrocytes had been detected. Data are expressed as the mean ratio of CFSE-labelled erythrocytes recovered from treated vs control livers.

Protein concentration

Soluble protein concentration was measured using Bio-Rad protein assay reagent according to the manufacturer's instructions. Concentrations were calculated with reference to a BSA standard curve (0–5 mg ml^{-1}).

Statistical analysis

All results are expressed as mean ± s.e.mean. Values to be compared were analysed for non-normality using a Shapiro–Wilk test. Student's unpaired t-test was used for comparing two groups when normality was indicated and Mann–Whitney U-test was used for non-parametric data. For multiple comparisons between three or more groups,

one-way ANOVA with Scheffé comparison was used for parametric data and Kruskall–Wallis (Conover–Inman) sta-tistical test for non-parametric data. All calculations were performed using StatsDirect statistical software (Research Solutions, Cambridge, UK); results were considered to be significant when P-values were less than 0.05.

Drugs, chemical reagents and other materials

Prazosin and terazosin were purchased from Sigma (Poole, UK), doxazosin and tamsulosin were kind gifts from Pfizer Global Research and Development (Sandwich, Kent, UK). ThermoTrace Infinity ALT liquid stable reagent was pur-chased from Alpha Labs (Eastleigh, UK). The protein assay reagent was purchased from Bio-Rad (Hemel Hempstead, UK), Gill 3 haematoxylin and ParaPlast Plus Wax were obtained from ThermoShandon (Cheshire, UK), 10% neutral buffered formalin was obtained from Surgipath Ltd (Peter-borough, UK). UltraPURE TRIzol reagent and PCR primers were obtained from Gibco BRL (Life Technologies, Paisley, UK). Reverse transcription kit, T4 polynucleotide kinase (10 U) and $10\times$ T4 polynucleotide kinase buffer was purchased from Promega (Southampton, UK). Ultima Gold scintillation fluid was purchased from Packard Biosciences (MA, USA). CFSE and TRITC dyes were purchased from Invitrogen (Paisley, UK). All other reagents were of analytical or molecular grade and were supplied by Sigma.

Results

Plasma catecholamine levels are elevated following paracetamol administration

Plasma adrenaline and noradrenaline levels were signifi-cantly elevated 5 h post-paracetamol administration (Figure 1). The administration of prazosin 1 h before paracetamol treatment significantly reduced circulating noradrenaline and adrenaline levels, which remained similar to vehicle control values (Figure 1).

Antagonism of α_1-adrenoceptors prevents paracetamol-induced hepatotoxicity

To investigate the role of α_1-adrenoceptors in paracetamol-induced toxicity, prazosin was administered pre- and post-paracetamol. Serum ALT activity levels were measured as an indicator of hepatic damage at 1, 5 and 24 h post-dosing. Hepatotoxicity was deemed significant when ALT levels exceeded $200\,U\,l^{-1}$ and were statistically different from controls (Kitteringham et al., 2000). Administration of para-cetamol to CD-1 mice resulted in severe hepatotoxicity, reflected in significantly elevated ALT activity at 5 and 24 h (Figure 2). Pretreatment with prazosin prevented paracetamol toxicity at 5 h, with ALT levels remaining comparable to controls. Serum sampled at 24 h suggested that a single dose of pretreatment with prazosin was insufficient to completely protect against paracetamol-induced hepatotoxicity, at this later time. Multiple doses of prazosin (mPZ; see Materials and methods) were required to prevent the paracetamol-induced increase in ALT activity at 24 h (mean ALT $U\,l^{-1}\pm$ s.e.mean;
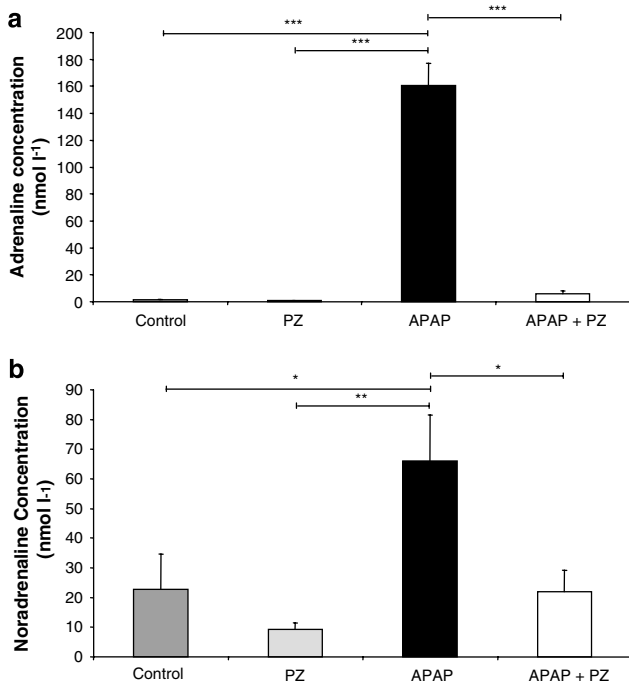
Figure 1 Plasma adrenaline and noradrenaline levels 5 h after giving paracetamol (APAP) ± prazosin (PZ) pretreatment. Mice were administered vehicle control, prazosin ($35.7\,\mu mol\,kg^{-1}$), paracetamol ($3.5\,mmol\,kg^{-1}$) or prazosin 1 h before paracetamol. Blood was collected and plasma was separated for HPLC analysis. (**a**) Results are mean adrenaline concentrations expressed as $nmol\,l^{-1} \pm$ s.e.mean, $n = 4$. ANOVA statistical analysis with Scheffé comparisons was used. ***$P < 0.001$. (**b**) Results are mean noradrenaline concentrations expressed as $nmol\,l^{-1} \pm$ s.e.mean, $n = 4$. ANOVA statistical analysis with Scheffé comparisons was used. *$P < 0.05$, **$P < 0.01$.

vehicle control: 21 ± 4; mPZ alone: 27 ± 6; paracetamol alone: 1229 ± 398; paracetamol + mPZ: 35 ± 9; $P < 0.001$ paracetamol vs vehicle control and mPZ alone, $P < 0.05$ paracetamol P vs paracetamol + mPZ). Prazosin alone did not raise ALT levels above $200\,U\,l^{-1}$ at any of the time points investigated.

Mice were also administered prazosin, post-paracetamol administration to assess the timescale of protection with regard to the onset of paracetamol-induced injury. Serum ALT activity levels indicated that prazosin significantly reduced hepatic damage when given 30 min after paracetamol. However, when prazosin was given 1 h after paracetamol, the reduction in ALT activity failed to reach statistical significance ($P = 0.08$; Figure 2b).

Histopathological examination of H&E-stained hepatic sections from treated animals

Histological analysis was performed to confirm prazosin-mediated hepatoprotection. H&E-stained hepatic sections from multiple vehicle controls and multiple prazosin-treated animals were microscopically normal (grade 0; Figure 3a and b, respectively). In agreement with the serum ALT data, livers taken 24 h after paracetamol displayed severe multi-focal, haemorrhagic necrosis extending from the centrilobular region to portal areas and adjacent lobules (Figure 3c). Parenchymal preservation was observed in the prazosin pretreated livers, with all the livers displaying normal
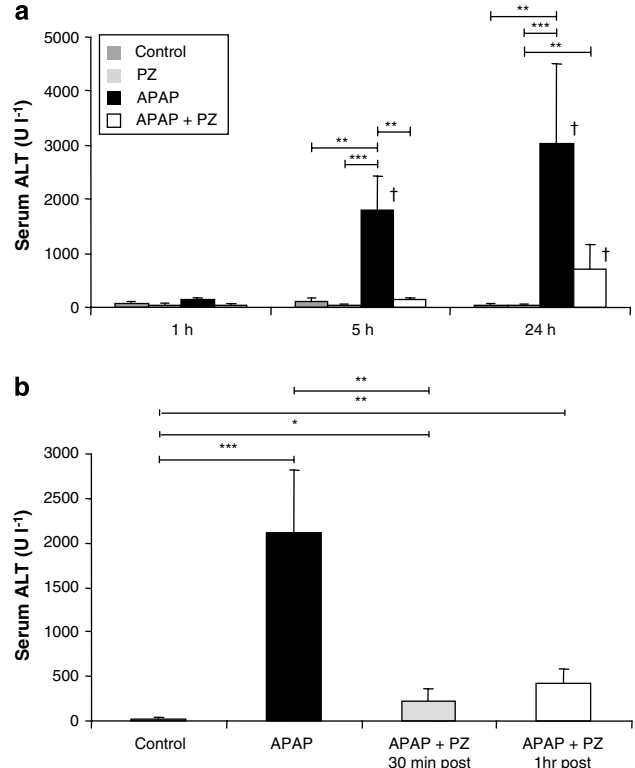

Figure 2 Effects of prazosin (PZ) on paracetamol (APAP)-induced hepatotoxicity at 1, 5 and 24 h. Serum alanine transaminase (ALT) levels were determined using Infinity ALT reagent. (**a**) Male CD-1 mice were dosed for 1, 5 and 24 h following prazosin pretreatment. Mice were administered vehicle control, prazosin ($35.7\,\mu mol\,kg^{-1}$), paracetamol ($3.5\,mmol\,kg^{-1}$) or prazosin 1 h before paracetamol. Results are expressed as mean serum ALT $U\,l^{-1} \pm$ s.e.mean, $n = 8–12$. (**b**) Male CD-1 mice were dosed with prazosin ($35.7\,\mu mol\,kg^{-1}$) 30 min and 1 h post-paracetamol ($3.5\,mmol\,kg^{-1}$; 5 h). CD-1 mice received vehicle control, paracetamol, paracetamol + prazosin 30 min post and paracetamol + prazosin 1 h post. Results are mean ALT $\pm$ s.e.mean, $n = 4$. One-way ANOVA with Scheffé comparison statistical analysis for normally distributed data and Kruskall–Wallis analysis for non-normally distributed data were used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, when compared with time-matched controls; $^{\dagger}P < 0.05$ when comparing identical treatment to values at 1 h.

histology (grade 0; Figure 3d). The centrilobular hepatocytes were observed to be paler and larger within this treatment group, with fewer intercellular spaces, suggesting hypertrophy, possibly to compensate for damaged hepatocytes.

Prazosin does not suppress paracetamol-induced GSH depletion

Hepatic GSH levels were rapidly and extensively depleted by paracetamol alone and in combination with prazosin (Figure 4). Replenishment of hepatic GSH was evident at 5 h within both the paracetamol-treated groups. By 24 h there were no discernible differences in GSH levels between dosing groups, despite the severity of the liver damage induced by paracetamol. Prazosin given alone failed to deplete GSH at any of the time points.

Prazosin does not alter paracetamol GSH metabolite formation

APAP–glutathione conjugate formation was monitored to determine whether prazosin was altering the elimination of
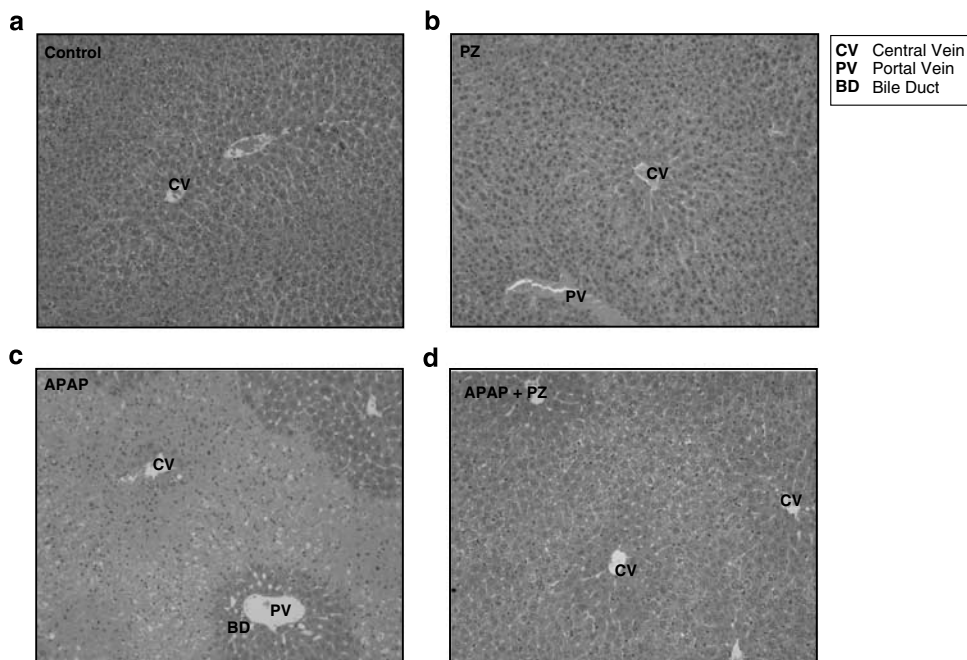
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Figure 3 Representative haemotoxylin and eosin (H&E)-stained liver sections from mice that had received paracetamol (APAP) and/or multiple corn oil (24 h). CD-1 mice were administered multiple corn oil vehicle control (**a**), prazosin (PZ; 35.7 μmol kg^{-1}) in corn oil at 90-min intervals up to five doses during the dosing period (**b**), paracetamol (3.5 mmol kg^{-1}) (**c**) and paracetamol + multiple prazosin (**d**) ($\times 10$ objective lens magnification).
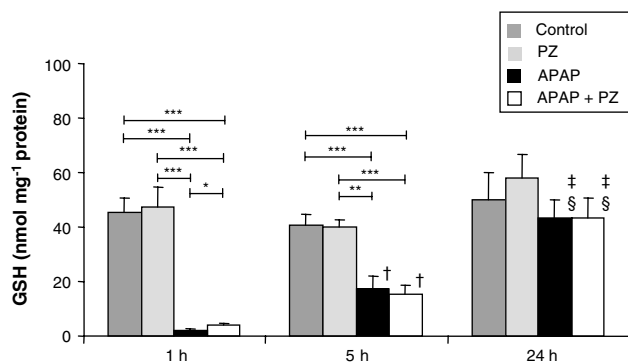

Figure 4 Effects of prazosin (PZ) on decreased hepatic glutathione (GSH) following paracetamol (APAP)-induced hepatotoxicity at 1, 5 and 24 h. GSH levels were determined according to the method of Vandeputte et al. (1994). Male CD-1 mice were dosed for 1, 5 and 24 h following a single prazosin pretreatment. Mice were administered vehicle control, prazosin (35.7 μmol kg^{-1}), paracetamol (3.5 mmol kg^{-1}) or prazosin 1 h before paracetamol. Results are expressed as mean GSH levels ± s.e.mean, $n = 4$–8. One-way ANOVA with Scheffé comparison analysis for normally distributed data and Kruskall–Wallis analysis for non-normally distributed data were used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, $^\dagger P < 0.05$, $^\ddagger P < 0.001$ vs 1 h, $^\S P < 0.01$ vs 5 h.

paracetamol and NAPQI. There was no difference in the recovery of hepatic paracetamol–SG conjugate 1 h after receiving paracetamol ± prazosin (% dose recovered ± s.e.mean; paracetamol 11.03 ± 1.83, paracetamol + prazosin 14.58 ± 2.00). Data previously generated within this laboratory has also confirmed that prazosin has no effect on paracetamol–glucuronide formation and therefore is unlikely to alter elimination via phase II metabolism (Clement and Williams, 2005).

Prazosin does not alter the level of paracetamol irreversibly bound to hepatic proteins

Covalent modification of critical cellular proteins has been shown to be important to the development of paracetamol-induced toxicity (Park et al., 2005). [^{3}H]paracetamol irreversibly bound to hepatic proteins was assessed following the removal of unincorporated radiolabel by extensive solvent extraction. There was no difference observed in the mean percentage of [^{3}H]paracetamol bound per milligram protein in prazosin pretreated (0.22 ± 0.05) vs paracetamol alone at 1 h (0.25 ± 0.10; $n = 10$ throughout these assays). Covalent binding increased significantly with the onset of toxicity during the 5 h experimental period. A similar amount of [^{3}H]paracetamol was bound to hepatic proteins in both treatment groups (% bound per milligram protein ± s.e.mean, paracetamol 1.41 ± 0.23 vs paracetamol ± prazosin 1.30 ± 0.39), indicating that prazosin was not altering the ability of NAPQI to become covalently bound.

Prazosin does not induce hepatocellular defence genes to prevent paracetamol-induced toxicity

Hepatic HO-1 and GCLC gene expression levels were investigated as biomarkers of hepatocellular defence. There were no statistical differences in GCLC expression at 1 h in any of the treatment groups (1 h GCLC/GAPDH ratio ± s.e.mean; vehicle control 0.54 ± 0.07, prazosin 0.38 ± 0.09, paracetamol 0.41 ± 0.12, paracetamol + prazosin 0.39 ± 0.02; $n = 6$ throughout). Paracetamol significantly increased GCLC mRNA levels at 5 h in an attempt to replenish GSH lost during bioactivation (5 h GCLC/GAPDH ratio ± s.e.mean; vehicle control 0.40 ± 0.04, prazosin 0.47 ± 0.10, paracetamol 0.71 ± 0.13, paracetamol + prazosin

0.45 ± 0.03; $P < 0.01$ paracetamol vs control, $P < 0.01$ paracetamol vs prazosin, $P < 0.05$ paracetamol vs paracetamol + prazosin). When dosed in combination, prazosin attenuated paracetamol-induced GCLC upregulation.

Similar findings were observed with HO-1 mRNA levels, which remained at control levels in all treatment groups 1 h post-dosing (1 h HO-1/cyclophilin ratio ± s.e.mean; vehicle control 0.52 ± 0.04, prazosin 0.41 ± 0.04, paracetamol 0.51 ± 0.03, paracetamol + prazosin 0.45 ± 0.06; $n = 6$ throughout). Mice that received paracetamol alone for 5 h showed a significant induction in HO-1 levels, which was prevented with the prazosin pretreatment (5 h HO-1/cyclophilin ratio ± s.e.mean; vehicle control 0.48 ± 0.08, prazosin 0.47 ± 0.11, paracetamol 1.44 ± 0.10, paracetamol + prazosin 0.60 ± 0.13; $P < 0.001$ paracetamol vs control, $P < 0.001$ paracetamol vs prazosin, $P < 0.001$ paracetamol vs paracetamol + prazosin). Prazosin alone did not alter HO-1 mRNA levels at either time point.

Prazosin prevents paracetamol-induced hepatic congestion
Labelled erythrocyte recovery from the livers of treated animals was used as an indicator of hepatocellular congestion and blood flow stagnation. Fluorescence-activated cell sorting analysis was used to determine the ratio of CFSE-labelled erythrocytes recovered from 5 h treated liver compared with the TRITC-labelled erythrocyte internal standard (Figure 5a–d, region 1). A significantly larger population of CFSE-labelled erythrocytes was recovered from paracetamol-treated livers (Figure 5c, region 4) when compared with other treatment groups (Figure 5e).

Other α-adrenoceptor antagonists also provide protection from paracetamol-induced hepatocellular damage
The structurally related adrenoceptor antagonists, doxazosin and terazosin, and the structurally unrelated adrenoceptor antagonist, tamsulosin, were administered 1 h before paracetamol to CD-1 mice. Doxazosin and terazosin also prevented the paracetamol-induced rise in ALT activity at 5 h (Figure 6a–c). Tamsulosin significantly reduced serum ALT activity after paracetamol administration; however, ALT levels were significantly different from controls and exceeded the toxic threshold, suggesting mild hepatocellular damage and incomplete protection.

Doxazosin and terazosin alone had no significant effects on hepatic GSH levels (Figure 6d–e). However, neither doxazosin nor terazosin prevented the depletion of GSH attributable to paracetamol. Similarly, tamsulosin alone had no effect on hepatic GSH at 5 h but when given in combination with paracetamol, GSH levels were significantly depleted to the same level as after paracetamol alone (Figure 6f).

Discussion and conclusions

Acute liver failure due to paracetamol overdose is a significant clinical problem and could benefit from new therapeutic strategies. Understanding the pathophysiological processes that underlie paracetamol-induced toxicity is an integral part of devising such strategies. In this study, using the mouse as an established experimental model, we provide evidence of a role for α-adrenoceptors in paracetamol-mediated hepatotoxicity in mice. Our data demonstrate that the induction of paracetamol-mediated toxicity is accompanied by increased levels of circulating catecholamines. Chemicals such as bromobenzene and CCl_4 have previously been shown to alter circulating catecholamine levels. It has been demonstrated that serum adrenaline levels peak within 2 h of CCl_4 administration, which occurred before significant toxicity had developed (Rubenstein, 1962). Bromobenzene elevated serum adrenaline, but not noradrenaline levels in mice at 4 h, which coincided with the bromobenzene-induced depletion of GSH prior to the onset of toxicity (Kerger *et al.*, 1988a).

Our observations led us to investigate whether antagonists of adrenoceptors could prevent or reverse the toxicity mediated by paracetamol, when given either before or after the hepatotoxin. We were able to confirm that the α_1 antagonist, prazosin was able to completely protect mice against paracetamol toxicity. Toxicological analysis revealed that prazosin could provide protection against a toxic, sublethal paracetamol dose for up to 5 h but offered only incomplete protection with a single dose at 24 h. Multiple doses of antagonist, administered during the first 6 h, as previously described for hepatotoxic modulation with other adrenergic antagonists (Kerger *et al.*, 1988a), were required to maintain complete protection for 24 h, consistent with the short half-life of the α-blocker in this species.

α-Adrenoceptor antagonists of pharmacological and chemical classes, related and unrelated to prazosin were equally capable of attenuating paracetamol toxicity. Doxazosin and terazosin were able to suppress ALT activity at 5 h; however, there was evidence of minor ALT release with the α_{1A} subtype-selective antagonist tamsulosin, indicative of mild hepatocellular damage at 5 h. This reduction of protection may be related to the relative affinities of these drugs for the adrenoceptor subtypes. Transgenic mice have revealed that the α_{1B}-adrenoceptors play a role in the pressor response to phenylephrine (Cavalli *et al.*, 1997). The murine liver contains an almost homogenous population of α_{1B} receptors (Garcia-Sainz *et al.*, 1995; Yang *et al.*, 1998), in contrast to the human liver that contains predominantly α_{1A}-adrenoceptors. It is thus possible that tamsulosin may be more effective in protecting against hepatotoxicity in man. However, prazosin would still be predicted to offer protection as it has sub-nanomolar affinity for α_{1A}, α_{1B} and α_{1D} subtypes.

Protection was not afforded by simply altering paracetamol metabolism or by inducing a hepatic defence response to overcome the hepatocellular insult. The progression of toxic liver injury is thought to involve inflammatory mediators (Laskin *et al.*, 1986; Michael *et al.*, 1999). Use of high-throughput cytokine protein arrays also revealed that prazosin was not preventing toxicity by modulating the cytokine-mediated progression of toxicity (see Supplementary data). Paracetamol intoxication initiated an immune response within the liver at 5 h, as cytokine and chemokine expression levels were elevated. Generally, these levels were diminished in paracetamol + prazosin-treated mice possibly due to the prevention of toxicity and hepatic damage.
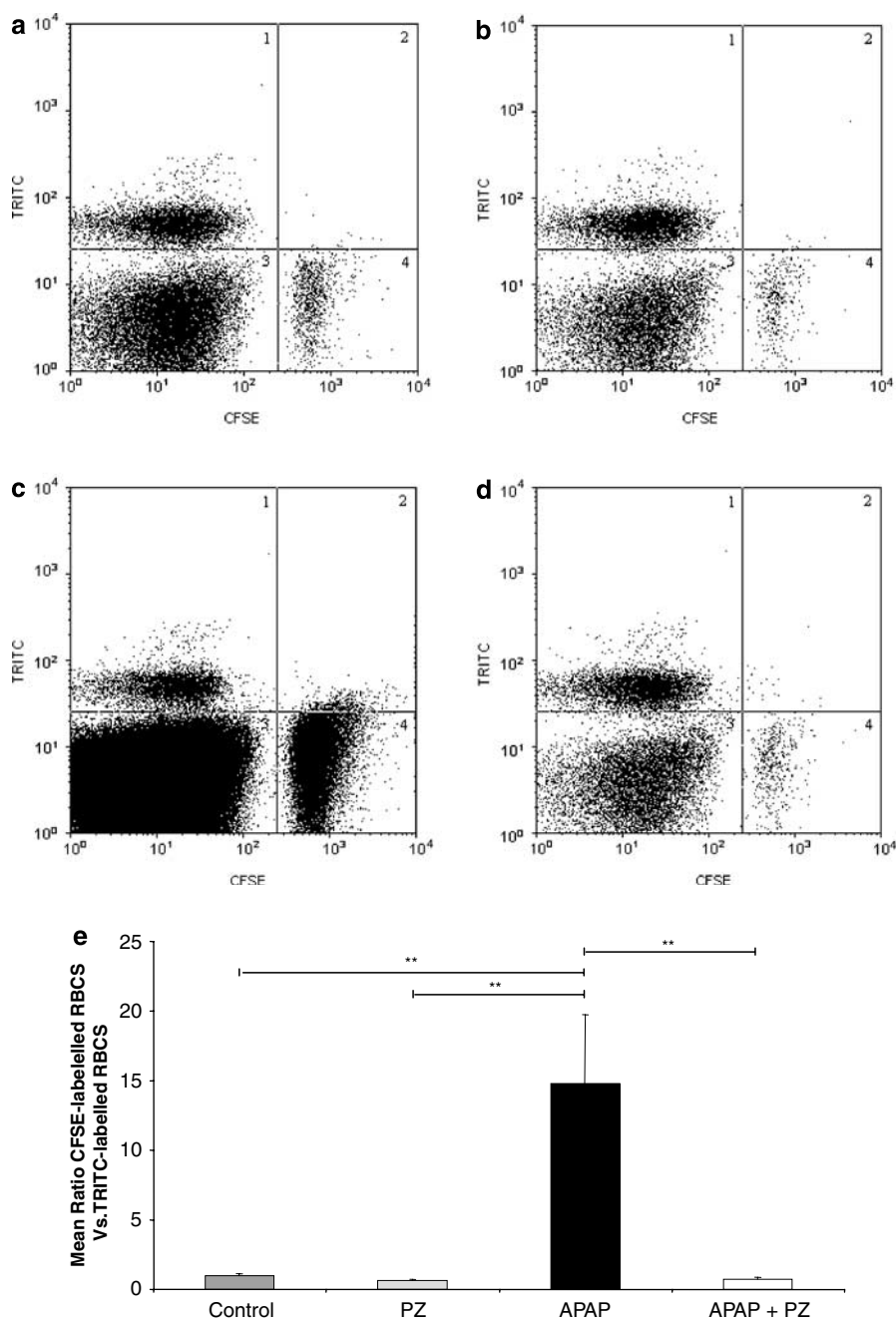
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Figure 5 Effect of prazosin (PZ) on carboxyfluorescein diacetate succinimidyl ester (CFSE)- and tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC)-labelled hepatic red blood cells (RBCs) recovered from paracetamol (APAP)-treated mice at 5 h. CFSE-labelled RBCs were administered i.v. to male CD-1 mice 16 h before paracetamol ± prazosin treatment. Mice were then given either vehicle control (**a**), prazosin (35.7 μmol kg^{-1}; **b**), paracetamol (3.5 mmol kg^{-1}; **c**) or prazosin 1 h before paracetamol (**d**) for 5 h. Representative dot plots of RBCs recovered from the livers of treated mice after 5 h are shown. TRITC-labelled RBCs are shown in region 1 with CFSE-labelled RBCs in region 4. RBCs were assayed using fluorescence-activated cell sorting analysis, CFSE-labelled cells were counted until 4000 TRITC-labelled cells had been counted (**e**). Results are expressed as mean ratio of CFSE-labelled RBCs vs TRITC-labelled RBCs, $n = 6$. ANOVA statistical analysis with Scheffé comparisons was used. **$P < 0.01$.

Prazosin did not provide protection by inhibition of bioactivation to NAPQI, as depletion of hepatic GSH levels was unaltered by prazosin pretreatment. Equally, analysis of hepatic paracetamol–protein adducts indicated that similar levels of covalent modification were observed between treatment groups regardless of prazosin pretreatment. α_1-Adrenoceptor antagonism did not assist paracetamol bio-inactivation by increasing conjugation to GSH. There

was also no upregulation of hepatic defence genes following prazosin pretreatment, suggesting that defence genes are upregulated as a consequence of toxicity and are not the mechanism of hepatoprotection.

We hypothesized that prazosin was providing protection through its pharmacological effect on haemodynamic regulation. Within the liver, it was demonstrated that paracetamol-induced toxicity was accompanied by a
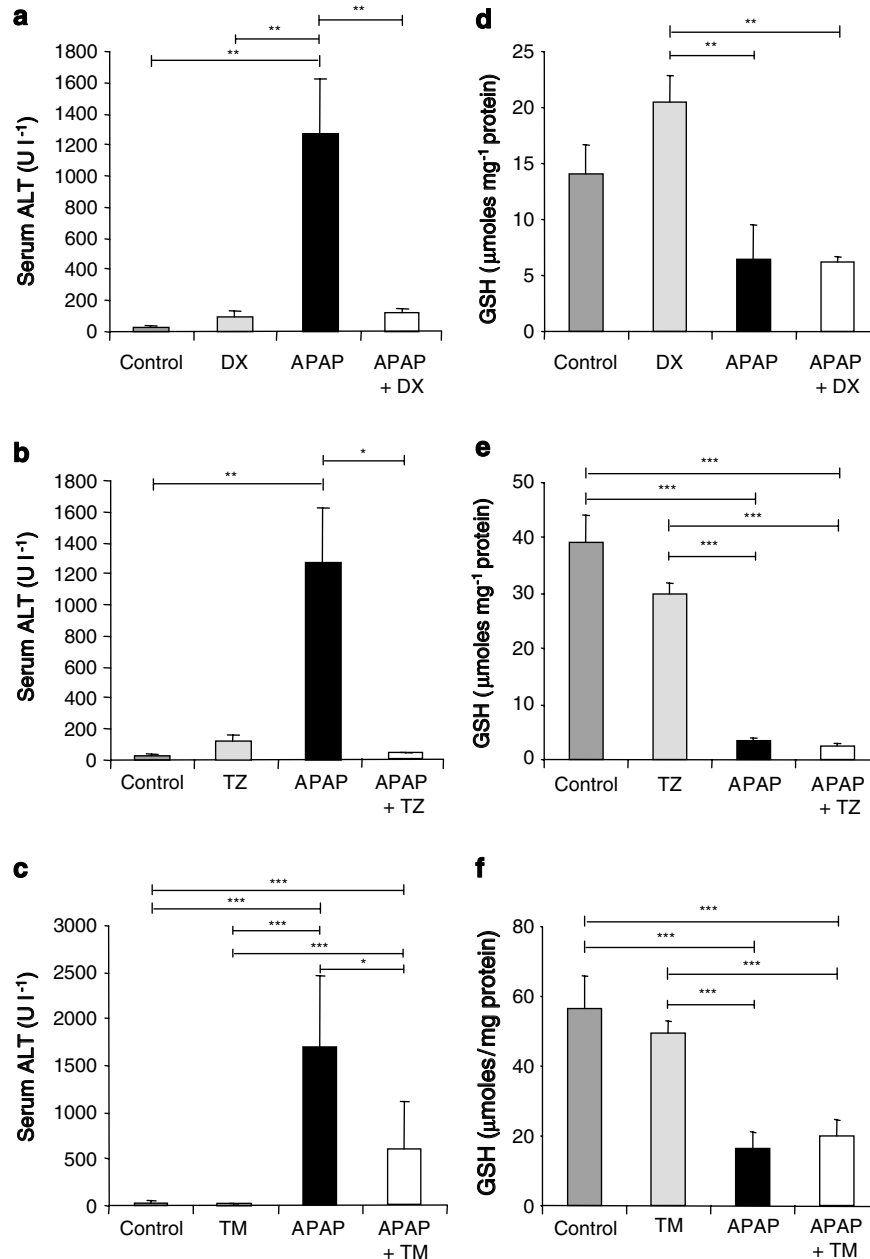
Figure 6 The effect of doxazosin (DX), terazosin (TZ) and tamsulosin (TM) on 5 h serum alanine transaminase (ALT) activity and hepatic glutathione (GSH) levels following paracetamol (APAP)-induced hepatotoxicity. Serum ALT levels were determined using Infinity ALT reagent (**a–c**). Hepatic GSH was determined according to the method of Vandeputte *et al.* (1994) (**d–f**). Male CD-1 mice were administered vehicle control, α antagonist (35.7 μmol kg^{-1}), paracetamol (3.5 mmol kg^{-1}) or α antagonist 1 h before paracetamol. DX (**a, d**), terazosin (**b, e**) and tamsulosin (**c, f**). Results are expressed as either mean serum ALT U l^{-1} ± s.e.mean or mean GSH ± s.e.mean, $n = 4$–12. One-way ANOVA with Scheffé comparison analysis for normally distributed data and Kruskall–Wallis analysis for non-normally distributed data were used. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

perturbation of the hepatic microcirculation. Histopathological analysis revealed that toxic livers were congested, with a profound accumulation of erythrocytes within the sinusoids. This is consistent with previous studies where paracetamol-induced microvascular disturbances, such as reduced sinusoidal perfusion and congestion, have been observed (Walker *et al.*, 1980; Bauer *et al.*, 2000; Coen *et al.*, 2003; Ito *et al.*, 2003). To quantify the degree of microvascular circulatory stasis, we developed a method that utilizes fluorescently labelled erythrocytes as markers of

erythrocyte accumulation within the liver. Using this methodology, it was observed that paracetamol toxicity is associated with a significant increase in the number of CFSE-labelled red blood cells (RBCs) trapped within the liver. This signified a compromise in the microvascular circulation induced by paracetamol toxicity. The administration of prazosin prevented such a microcirculatory dysfunction, observed through the lack of RBC accumulation, analysed by histology as well as through the use of labelled erythrocytes. Maintenance of the hepatic microcirculation in the presence

of a continuing toxic insult may be critical to halt the progression of paracetamol toxicity. It is possible that microcirculatory stasis and RBC accumulation within the sinusoids are due to catecholamine-induced constriction of the hepatic central veins. The ability of prazosin to prevent vasoconstriction and lower portal pressure through a reduction in intra-hepatic vascular resistance (Cummings et al., 1988; Albillos et al., 1994, 1995) would help to maintain an adequate blood flow to the liver during xenobiotic bioactivation and the development of toxicity. The delivery of oxygen, amino acids, cofactors and substrates to the liver would aid GSH replenishment, the maintenance of mitochondrial function and energy production to facilitate repair and regeneration, consistent with our findings. This would suggest that prevention of hepatic microvascular constriction is critical for preventing toxicity. Recent studies by Liu et al. (2003) and Fiorucci et al. (2004) demonstrate that agents such as NO donors that support vasodilation help to improve haemodynamics and provide hepatoprotection from paracetamol toxicity.

In view of our findings, we propose that paracetamol-induced toxicity requires the following sequence of events to occur. First, endogenous catecholamines are released as a consequence of paracetamol-induced hepatocellular damage. This is likely to be due to a physiological stress response elicited as a result of afferent signals arising from nerve endings within the liver. The precise innervation of the hepatic infrastructure is not well documented. However, studies have demonstrated extensive parenchymal innervation, with presumptive sensory nerve endings, and it has been postulated that adrenergic, cholinergic and peptidergic neurons register information from osmoreceptors, baroreceptors, ionic receptors, metabolic receptors and nociceptive receptors (Hall and Leach, 1999; Hartmann and Beckh, 1992). The liver capsule is a visceral structure with afferent input into the central nervous system, probably routed via the sympathetic nervous system (Hall and Leach, 1999). It has been noted that data from animal studies suggest that excessive afferent activity after chemical irritation may be assimilated and interpreted as pain (Hall and Leach, 1999). Second, raised catecholamine levels cause hepatic microvascular vasoconstriction, leading to a compromise in the perfusion of the hepatic microvascular bed. Third, poor perfusion coupled with direct cellular toxic effects causes hepatocyte dysfunction and ultimately necrosis. Finally, this sets up a positive feedback mechanism resulting in amplification of local damage to include the whole organ. Prazosin may thus interrupt the earliest stages of this toxicity cycle by maintaining vascular integrity, thereby preventing sinusoidal congestion, blood stasis and the ensuing cascade of ischaemic hypoxia preventing the progression of toxic damage.

Our findings indicate that prazosin administration significantly modifies the hepatic response to paracetamol intoxication, which may have implications for the therapeutic management of ALF due to paracetamol overdose. Current treatment regimens depend on the repletion of hepatic GSH stores with N-acetylcysteine. Treatment with N-acetylcysteine may be used up to 24 h after ingestion of paracetamol; however, the maximum protective effect is obtained up to 8 h post-ingestion (Prescott et al., 1979). The effectiveness of this antidote declines sharply after this time. A significant proportion of patients are therefore lost due to the inability to initiate N-acetylcysteine administration within the therapeutic time window (Prescott et al., 1979; Wallace et al., 2002). The use of additional agents that could be administered at later times would be of considerable clinical advantage. It is tempting to speculate that provision of prazosin or other agents that maintain hepatic perfusion in conjunction with conventional treatments could extend the therapeutic time window and improve survival rates. Such potential adjuncts to conventional therapy could expand the range of therapeutic strategies currently at the disposal of the clinician. There is pressing need for such combination therapies to be tested and validated in animal models of paracetamol toxicity.

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Conflict of interest

The authors state no conflict of interest.

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