A Cytochrome P450–Independent Mechanism of Acetaminophen-Induced Injury in Cultured Mouse Hepatocytes[§]

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ABSTRACT

Mouse hepatic parenchymal cells (HPCs) have become the most frequently used *in vitro* model to study mechanisms of acetaminophen (APAP)-induced hepatotoxicity. It is universally accepted that APAP hepatocellular injury requires bioactivation by cytochromes P450 (P450s), but this remains unproven in primary mouse HPCs *in vitro*, especially over the wide range of concentrations that have been employed in published reports. The aim of this work was to test the hypothesis that APAP-induced hepatocellular death *in vitro* depends solely on P450s. We evaluated APAP cytotoxicity and APAP-protein adducts (a biomarker of metabolic bioactivation by P450) using primary mouse HPCs in the presence and absence of a broad-spectrum inhibitor of P450s, 1-aminobenzotriazole (1-ABT). 1-ABT abolished formation of APAP-protein adducts at all concentrations of APAP (0–14 mM), but eliminated cytotoxicity only at small

concentrations (≤ 5 mM), indicating the presence of a P450-independent mechanism at larger APAP concentrations. P450-independent cell death was delayed in onset relative to toxicity observed at smaller concentrations. *p*-Aminophenol was detected in primary mouse HPCs exposed to large concentrations of APAP, and a deacetylase inhibitor [bis (4-nitrophenyl) phosphate (BNPP)] significantly reduced cytotoxicity. In conclusion, APAP hepatocellular injury *in vitro* occurs by at least two mechanisms, a P450-dependent mechanism that operates at concentrations of APAP ≤ 5 mM and a P450-independent mechanism that predominates at larger concentrations and is slower in onset. *p*-Aminophenol most likely contributes to the latter mechanism. These findings should be considered in interpreting results from APAP cytotoxicity studies *in vitro* and in selecting APAP concentrations for use in such studies.

Introduction

Overdose with acetaminophen (APAP) is the leading cause of drug-induced liver injury and acute liver failure in the United States and other developed countries (Ostapowicz et al., 2002; Larson et al., 2005; Khashab et al., 2007; Fontana, 2008). As a result, there have been many studies of APAP hepatotoxicity with the ultimate goals of understanding mechanisms of liver injury and developing more effective preventive and therapeutic strategies for APAP overdose.

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Liver injury from APAP in mice resembles in most ways that which occurs in humans suffering from APAP overdose (McGill et al., 2012). For this reason, the mouse model *in vivo* and primary mouse hepatic parenchymal cells (HPCs) *in vitro* have become most frequently used for studies of mechanisms of APAP-induced hepatocellular injury. The pioneering work of Dr. Bernard Brodie and coworkers (Jollow et al., 1973; Mitchell et al., 1973a,b; McGill and Jaeschke, 2013) in mice in the mid-1970s and subsequent studies by others have led to universal acceptance that APAP hepatotoxicity requires bioactivation to *N*-acetyl-*p*-quinoneimine (NAPQI) by cytochrome P450 enzymes (P450s). It seemed reasonable to assume that hepatocellular death from APAP *in vitro* also requires bioactivation by P450s, and many studies of mechanisms have relied upon this assumption. Nonetheless, the assumption has not been tested.

A PubMed search revealed that investigators using isolated primary mouse HPCs have employed a wide range of APAP concentrations, that is, 0.1–50 mM, with 5 and 10 mM used

ABBREVIATIONS: ABAM, antibiotic-antimycotic; 1-ABT, 1-aminobenzotriazole; ALT, alanine aminotransferase; APAP, acetaminophen; BNPP, bis (4-nitrophenyl) phosphate; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; GSH, glutathione; HPC, hepatic parenchymal cell; MRM, multiple reaction monitoring; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; NAT, *N*-acetyltransferase; P450, cytochrome P450; PAP, *p*-aminophenol; PAP-SG, 4-amino-3-*S*-glutathionylphenol; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TMCS, trimethylchlorosilane; TMS, trimethylsilyl; WME, Williams' Medium E.

Fig. 1. APAP concentrations used in published studies of murine HPC cytotoxicity in vitro. A PubMed search was conducted in October 2014, using the key words “acetaminophen” and “mice” and “hepatocyte.” Perusal of the abstracts and publications dating back to 1978 revealed 60 peer-reviewed reports that used primary mouse HPCs in the study of APAP cytotoxicity. Some papers employed several concentrations. The number of publications employing each concentration is plotted as a frequency diagram.

most commonly (Fig. 1). Concentrations exceeding 10 mM were used as early as 1991 and as recently as 2013. In mice treated in vivo, the most typical doses of APAP used to produce hepatotoxicity are 300 or 400 mg/kg; plasma concentration at a dose of 400 mg/kg would not be expected to exceed 3 mM [maximum plasma concentration = dose/ V_d = (400 mg/kg ÷ 151 mg/mmol)/0.9 l/kg = 2.9 mM]. Based on this calculation, concentrations of 5 mM or less would seem reasonable for in vitro studies. The use of concentrations greater than 5 mM raises the question as to whether P450-mediated metabolism of APAP to NAPQI is the initiating mechanism at these larger concentrations. Accordingly, we undertook a concentration-response study in primary mouse HPCs to evaluate cytotoxicity in the presence and absence of an inhibitor of P450-mediated bioactivation of APAP. The results pointed to a P450-independent mechanism that predominates in vitro at larger APAP concentrations.

Materials and Methods

1-Aminobenzotriazole (1-ABT), bis (4-nitrophenyl) phosphate (BNPP), 4-aminophenol [*p*-aminophenol (PAP)], and collagenase from *Clostridium histolyticum* were purchased from Sigma-Aldrich (St. Louis, MO). Antibiotic-antimycotic (ABAM), Dulbecco's phosphate-buffered saline (PBS), liver perfusion medium, hepatocyte wash medium, fetal bovine serum, L-glutamine, and Williams' Medium E (WME) were purchased from Life Technologies (Carlsbad, CA). Alanine aminotransferase (ALT) reagent was purchased from Thermo Scientific (Pittsburgh, PA). Type I collagen from rat tail was purchased from BD Biosciences (San Jose, CA). Bovine insulin for cell culture was purchased from Cell Applications (San Diego, CA). Triton X-100 was purchased from Research Products International (Mount Prospect, IL).

Animals. Wild-type C57BL/6J, male mice (9 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME). They were acclimated for at least 1 week at 22°C with alternating 12-hour light/dark cycle and access to standard chow diet (Teklad 22/5 Rodent

Diet 8940; Harlan Laboratories, Indianapolis, IN) and spring water ad libitum until they were euthanized for cell isolation. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee.

Isolation and Treatment of Primary Mouse HPCs. Primary mouse HPCs were isolated, as described by Klaunig and coworkers with some modification (Klaunig et al., 1981; Bajt et al., 2004). Briefly, each mouse was anesthetized with 50 mg/kg pentobarbital i.p., after which the abdominal cavity was opened and a 24-gauge catheter was placed in the inferior vena cava and secured. The liver was perfused first with 50 ml liver perfusion medium supplemented with ABAM. The portal vein was severed, and the anterior vena cava was clamped with a hemostat. Then the liver was perfused with 40 ml WME supplemented with ABAM and 5–10 mg type IV collagenase. After the perfusion was completed, the liver was removed, placed in hepatocyte wash medium at room temperature, and gently broken apart with forceps. The cell mixture was then filtered through three layers of gauze and centrifuged at 50g for 2 minutes. Hepatocytes were washed two more times, and viability was evaluated with trypan blue exclusion; cell viability of $\geq 85\%$ was the criterion for use in experiments. The HPCs (1.25×10^5 cells/well) were plated in 24-well plates coated with type I collagen ($5 \mu\text{g}/\text{cm}^2$) from rat tail and cultured overnight in 0.5 ml WME supplemented with 10% fetal bovine serum and containing ABAM, 2 mM L-glutamine, and 1×10^{-7} M insulin and incubated at 37°C in 5% CO_2 . After 1 hour, medium was replaced (to remove unattached cells), and cells were incubated for another hour. Hepatocytes were then gently washed twice with PBS, followed by WME supplemented with ABAM and 2 mM L-glutamine. Inhibitor was added at this point when appropriate.

1-ABT was diluted with PBS (6.7 mg/ml), and the final concentration of 1-ABT in the medium was 1 mM. BNPP was diluted with PBS (5 mg/ml) and warmed in a water bath at 40–45°C; the final concentration of BNPP in the medium was 1 mM. One hour after addition of inhibitors, HPCs were washed with PBS once, APAP was added, and the cells were incubated for an additional 2.5–18 hours. APAP was dissolved in WME supplemented with ABAM and 2 mM L-glutamine. PAP was dissolved in PBS (1 mg/ml) and applied to HPCs cultured in WME to achieve final medium concentrations of 100–800 μM .

ALT Assay. Medium was collected, and remaining cells were lysed and collected by adding an equal volume of 1% Triton X-100 and scraping with a cell scraper. Medium and cell lysate were centrifuged separately at 660g for 5 minutes, and ALT activity in these fractions was measured using Infinity ALT reagent. ALT released into the medium was expressed as a percentage of total ALT activity (i.e., medium plus lysate). In the time course study, baseline ALT activity at each time point was subtracted from values obtained at each APAP concentration.

APAP-Protein Adducts. After 2.5 hours and 8 hours of APAP treatment, cells were removed using a cell scraper, and medium and cells of three replicate wells were combined, sonicated, and frozen at -80°C until analysis. APAP-protein adducts (APAP-cysteine) were detected by high-performance liquid chromatography with electrochemical detection, as previously reported (Muldrew et al., 2002). Briefly, samples were treated with protease and precipitated with trichloroacetic acid. The resulting supernatant was injected into the high-performance liquid chromatography column using a Model 582 solvent delivery system, and APAP-cysteine was detected with a Model 5600A CoulArray detector (ESA, Chelmsford, MA). The range of linearity for this method was 0.01–20 μM APAP-cysteine. The coefficients of variation for the assay were consistently $<10\%$ at concentrations of 0.03, 0.125, 0.5, and 2.0 μM APAP-cysteine adducts. Based on the coefficients of variation for the standard curve for the assay, the lower limit of quantification was 0.01 μM APAP-cysteine. APAP-cysteine content was reported relative to the protein content of the sample (nmol/mg protein); protein content was determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA).

Quantification of PAP. Analysis of PAP concentration in cultured cells was performed by gas chromatography tandem

quadrupole mass spectrometry. Specifically, 1 ml cultured HPCs in medium and 10 ml ethyl acetate were combined, vortexed, and then centrifuged. The solvent layer was passed through anhydrous sodium sulfate to remove excess water. This process was repeated, and extracts were combined. Samples were dried under nitrogen and then reconstituted with 100 μ l methylene chloride. Then, 50 μ l sample was transferred to an autosampler vial with a 250 μ l glass insert and 25 μ l BSTFA [*N,O*-bis(trimethylsilyl) trifluoroacetamide] + TMCS (trimethylchlorosilane; 99:1) and heated to 70°C for 30 minutes prior to analysis for the formation of trimethylsilyl (TMS) derivatives. PAP quantification was based on the area of the PAP-TMS m/z (mass-to-charge ratio) 253.1→238.1 multiple reaction monitoring (MRM) transition as interpolated on a quadratic standard curve constructed from 0.1, 0.2, 0.5, 1.0, and 5.0 μ g/ml standards for PAP reacted with 1% TMCS. Calibration curve R^2 values were >0.999. PAP-TMS retention times were consistent at an average of 10.37 minutes $\pm$ 0.07 minute (to 1 S.D.). Carryover to solvent blanks was essentially nonexistent, measured at only 0.2%. Compound identity was confirmed not only by retention time, but also by determination of the ratio of the MRM m/z 253.1→238.1 area to those of two additional MRM settings, m/z 238.1→222.1 and m/z 238.1→147.1. These were consistently within 20% uncertainty limits and averaged 43.3% ($\pm$ 3.3%) and 32.3% ($\pm$ 2.4%), respectively. Based on the blank method of calculating limits of detection and limits of quantification (Swartz and Krull, 1997), the limit of detection was 0.00095 μ g/ml and the limit of quantification was 0.0032 μ g/ml.

Statistical Analysis. Data are expressed as mean $\pm$ S.E.M. Data that were not normally distributed were subjected to appropriate transformation (i.e., logarithmic, square root, arcsin squared). Student's *t* test was used to compare two means, and analysis of variance was used when more than two means were compared. If a difference was detected with analysis of variance, Holm-Sidak post hoc testing was performed. If data transformation failed to generate data with a normal distribution, nonparametric tests were performed (Mann-Whitney *U* test for comparison of two means and Kruskal-Wallis one-way analysis of variance for multiple comparisons). *P* < 0.05 was set as the criterion for statistical significance.

Results

Concentration Dependence of APAP Cytotoxicity In Vitro. A concentration-response study in primary mouse HPCs was performed to evaluate APAP cytotoxicity in vitro in the presence and absence of 1-ABT, a broad-spectrum, suicide inhibitor of P450s. In the absence of 1-ABT, ALT activity in the medium at 18 hours increased as APAP concentration

increased from 0.1 mM to 1.25 mM, reaching a plateau at about 40% ALT release (Fig. 2A). Beyond 5 mM APAP, cytotoxicity increased markedly with APAP concentration. ALT release was essentially complete (90%) at 12 mM APAP.

NAPQI binds covalently to cysteinyl residues on proteins, forming APAP-protein adducts. These adducts were measured 2.5 hours after addition of APAP as a marker of P450-dependent bioactivation of APAP to NAPQI. APAP-protein adducts increased in parallel with the increase in ALT activity up to 5 mM APAP, at which concentration they reached a plateau and showed no further concentration dependence (Fig. 2A).

1-ABT cotreatment eliminated the formation of APAP-protein adducts at all concentrations of APAP (Fig. 2B). The concentration of APAP adducts did not change between 2.5 and 8 hours, indicating cessation of APAP bioactivation within 2.5 hours, and 1-ABT provided effective inhibition at both of these times (Supplemental Fig. 1). With 1-ABT pretreatment, concentrations of 5 mM APAP or less produced no statistically significant increase in ALT activity in the medium (Fig. 2B). However, a concentration-dependent increase in ALT release was observed at APAP concentrations of 8 mM and greater, despite the lack of formation of APAP-protein adducts. A different P450 inhibitor, metyrapone, also afforded protection from cytotoxicity at small, but not large, APAP concentrations (Supplemental Fig. 2).

Development of APAP Cytotoxicity. Examination of ALT release over time revealed that at small APAP concentrations (i.e., up to 5 mM), ALT release into the medium increased within 6 hours to about 40%, but remained unchanged thereafter (Fig. 3A). In contrast, ALT activity continued to increase with time at larger APAP concentrations (i.e., 8 mM and above). In 1-ABT-pretreated cells, there was no significant ALT release at any concentration of APAP at 6 hours (Fig. 3B). Indeed, no significant ALT release occurred at any time in 1-ABT-pretreated HPCs at APAP concentrations of 5 mM or less. The cytotoxicity at larger APAP concentrations began only after 6 hours and progressed with time (Fig. 3B).

A Deacetylase Inhibitor Reduces APAP Cytotoxicity. The results above pointed to one or more P450-independent initiating mechanisms of HPC injury at large APAP concentrations in vitro. An intriguing alternative bioactivation

Fig. 2. Concentration-dependent ALT release and APAP-protein adduct appearance in HPCs exposed to APAP. Primary mouse HPCs were treated with various concentrations of APAP (0.1 to 14 mM) without (A) or with (B) 1-hour pretreatment with 1-ABT. ALT release was measured 18 hours after the APAP treatment, and APAP-protein adducts were measured at 2.5 hours. ^aSignificantly different from 0 mM APAP; ^bsignificantly different from 0.1 mM APAP; ^csignificantly different from concentrations of APAP $\leq$ 5 mM. *N* = 4.

Fig. 3. Time course of ALT release from primary mouse HPCs treated with APAP. Primary mouse HPCs were treated with various concentrations of APAP (0.1–14 mM) without (A) or with (B) 1-hour pretreatment with 1-ABT. ALT release was measured at 6 hours, 12 hours, and 18 hours. ^aSignificantly different from same APAP concentration at 6 hours; ^bsignificantly different from same APAP concentration at 12 hours. *N* = 4.

pathway is through deacetylation of APAP to form PAP and acetate (Nicholls et al., 1997; McConkey et al., 2009; Zhao and Pickering, 2011). Although PAP has not received attention as a contributor to APAP hepatotoxicity, administration of PAP to mice causes liver injury (Song and Chen, 2001). PAP has been implicated, however, in acute proximal renal tubular injury caused by APAP, both in animal models and in cultured renal cells. Newton et al. (1985a,b) presented evidence that reactive intermediate(s) of PAP that bind to kidney proteins initiates the renal toxicity of APAP in Fischer 344 rats, and that inhibition of APAP deacetylation by BNPP decreases covalent binding of radiolabeled APAP and reduces nephrotoxicity. This suggested that PAP is responsible for the renal toxicity of APAP in F344 rats.

With these observations as a backdrop, we examined the possibility that deacetylation to PAP contributes to the initiation of P450-independent APAP toxicity in isolated HPCs. Little PAP was detected in HPC cultures (i.e., medium plus cells) exposed to 2.5 mM APAP; however, in HPCs exposed to 14 mM APAP, a substantial increase in PAP concentration occurred (Fig. 4). 1-ABT treatment did not affect PAP formation at either 2.5 or 14 mM APAP (Fig. 4). The deacetylase inhibitor BNPP was without effect on production of PAP in HPC cultures exposed to 2.5 mM APAP, but reduced substantially the amount of PAP detected in HPCs exposed to 14 mM APAP (Fig. 4). BNPP by itself caused no cytotoxicity (Fig. 5A), and it did not influence cytotoxicity caused by 2.5 mM APAP (Fig. 5B). However, it reduced cytotoxicity caused by either 10 or 14 mM APAP (Fig. 5, C and

D). Interestingly, 1-ABT caused a further reduction in cytotoxicity in BNPP-treated cells exposed to 10 or 14 mM APAP (Fig. 5, C and D). Consistent with results presented in Figs. 2 and 3, 1-ABT treatment alone eliminated the cytotoxicity caused by 2.5 mM APAP, reduced toxicity at 10 mM, and was without effect at 14 mM (Fig. 5).

PAP Is Cytotoxic to Primary Mouse HPCs. PAP caused a dose-dependent increase in ALT release from HPCs in vitro at medium concentrations greater than 200 μ M (Fig. 6). 1-ABT pretreatment was without effect on cytotoxicity from exogenously added PAP (Supplemental Fig. 3).

Discussion

Throughout several decades of investigating APAP toxicity in vitro, it has been assumed that initiating mechanisms that pertain at small but toxic APAP concentrations (i.e., 5 mM or less) are the same as those operative at larger concentrations. In many studies, the rationale for using greater concentrations was not explained; however, in some studies, dimethylsulfoxide (DMSO) was used as a vehicle to dissolve agents (e.g., mitogen-activated protein kinase inhibitors) used in the investigation. Because DMSO can inhibit APAP-bioactivating P450s (Arndt et al., 1989), large APAP concentrations were used ostensibly to overcome the P450-inhibiting ability of DMSO vehicle (Gunawan et al., 2006; Du et al., 2013).

Finding no definitive evidence for the assumption that P450-mediated bioactivation of APAP initiates toxicity at larger concentrations, we undertook a concentration-response

Fig. 4. Concentration of PAP in primary mouse HPC cultures treated with APAP. HPCs were treated with 2.5 mM (left) or 14 mM (right) APAP. Eighteen hours later, medium with disrupted cells was collected for measurement of PAP, as described in *Materials and Methods*. *N* = 5–6. ^aSignificantly different from 1-ABT only.

Fig. 5. Effect of inhibition of P450 and/or inhibition of deacetylation on ALT release from HPCs treated with APAP. Primary mouse HPCs were treated with 0 (A), 2.5 (B), 10 (C), or 14 (D) mM APAP after 1-hour pretreatment with or without 1-ABT (P450 inhibitor) and/or BNPP (deacetylase inhibitor). ^aDifferent from -1-ABT/-BNPP; ^bdifferent from +1-ABT/-BNPP; ^cdifferent from -1-ABT/+BNPP. *N* = 5–6.

study in primary mouse HPCs to evaluate cytotoxicity in the presence and absence of a broad-spectrum, suicide inhibitor of P450-mediated APAP bioactivation. In the absence of 1-ABT, the concentration-response relationship for HPC injury as marked by ALT release was biphasic, raising the possibility that more than one mechanism was at play. Interestingly, the concentration of APAP-protein adducts, a marker of P450-mediated bioactivation of APAP, increased in a manner that matched the cytotoxicity up to 5 mM APAP, but increased no further with increasing APAP concentration. This result further suggested the existence of two initiating mechanisms *in vitro*, one operating at small APAP concentrations for which maximal cytotoxicity (ALT release) was about 40% and another that predominated at larger APAP concentrations and led to essentially 100% ALT release. P450 inhibition with 1-ABT prevented the production of APAP-protein adducts at all concentrations of APAP used and also eliminated cytotoxicity at APAP concentrations of 5 mM or smaller; however, the marked increase in cytotoxicity at larger APAP concentrations remained despite inhibition of P450-initiated APAP bioactivation. These results clearly pointed to at least two mechanisms that contribute to APAP cytotoxicity *in vitro*.

A time-course study revealed that the cytotoxic response at small APAP concentrations began early, but was essentially complete by 6 hours; however, injury continued to progress with time at larger concentrations. In contrast, when the P450-dependent component was eliminated by 1-ABT, the cytotoxicity at larger concentrations did not begin until after 6 hours, and it progressed with time thereafter. Together, these results point to the occurrence of a rapidly developing, P450-dependent mechanism of cell injury that predominates at smaller, cytotoxic APAP concentrations and that is limited both in degree and duration, as well as a later-developing, P450-independent mechanism that predominates at larger APAP concentrations and can result in complete cell killing *in vitro*.

Because NAPQI is formed strictly by P450-mediated metabolism, the P450-independent mechanism cannot be due to NAPQI. It makes sense that the P450-dependent cytotoxicity is limited in magnitude in light of reports that NAPQI can inactivate the P450s involved in its formation from APAP (Snawder et al., 1994). Hepatocellular heterogeneity in P450 concentrations or in NADPH cofactor supply might also contribute to this limitation of cytotoxicity,

Fig. 6. Concentration-dependent ALT release from primary mouse HPCs exposed to PAP. Primary mouse HPCs were treated with various concentrations of PAP (100 to 700 μ M), and ALT release was measured 18 hours later. *Significantly different from 0 μ M PAP.

because quantitative differences in P450s among HPCs could result in different degrees of NAPQI production. For example, HPCs in the periportal regions of liver lobules display smaller P450 concentrations than those in the centrilobular regions (Gooding et al., 1978; James et al., 1981). Such heterogeneity could result in P450-dependent death of some HPCs, but only sublethal stress in others at the same APAP concentration in vitro.

The existence of a P450-independent mechanism might help to explain previous results. For example, in studies employing primary mouse HPCs exposed in vitro to a large APAP concentration (i.e., 10 mM), knockout of receptor-interacting protein kinase 3 resulted in reduced cytotoxicity early, but not upon continued APAP exposure (Ramachandran et al., 2013). Similarly, treatment of HPCs in vitro with cyclosporin A provided protection early that disappeared upon continued exposure to 10 mM APAP (Kon et al., 2004, 2007). Our results suggest that the early protection could be due to interference with the rapidly acting, P450-dependent mechanism of cell death, whereas the cytotoxicity that occurs later involves a P450-independent mechanism(s).

One P450-independent mechanism that might contribute to APAP-mediated cytotoxicity involves PAP. Little is known about the acute hepatotoxicity of PAP. However, the acute renal toxicity of APAP has been attributed to formation of PAP (Newton et al., 1985a,b). PAP exposure in vivo or in vitro led to glutathione (GSH) depletion in kidney cells (Shao and Tarloff, 1996; Song et al., 1999; Harmon et al., 2005, 2006). An inhibitor of GSH biosynthesis protected rats from PAP-induced renal injury (Gartland et al., 1990; Song et al., 1999), and GSH depletion decreased PAP cytotoxicity in renal cells in vitro (Klos et al., 1992). These results point to a toxic GSH conjugate of PAP as a mediator of renal cell injury. Several toxic GSH conjugates, including 4-amino-3-S-glutathionylphenol (PAP-SG), were found in kidney cell preparations in vitro and in bile of PAP-treated rats (Fowler et al., 1991; Klos et al., 1992). PAP-SG was more toxic than PAP itself, suggesting further that GSH conjugate(s) might be responsible for toxicity of PAP (Fowler et al., 1993).

Other studies revealed that PAP can form a 4-aminophenoxy free radical that oxidizes to yield benzoquinoneimine, which can bind covalently to renal proteins (Fowler et al., 1993). Ascorbate afforded protection from PAP nephrotoxicity in vivo and in vitro (Fowler et al., 1993; Song et al., 1999; Hallman et al., 2000; Harmon et al., 2005, 2006), suggesting a role for a free radical metabolite. The possibility of redox cycling of a PAP metabolite was mentioned by Rankin et al. (1996), and evidence of lipid peroxidation in rat kidneys and kidney slices has been presented (Kanbak et al., 1996; Harmon et al., 2005, 2006). In LLCPK1 kidney cells exposed to PAP, generation of reactive oxygen species (ROS) and cytotoxicity occurred; however, ROS scavengers failed to provide protection, suggesting that ROS are not the main cause of PAP-induced cytotoxicity in these cells (Foreman and Tarloff, 2008).

Although PAP has not received attention as a contributor to APAP hepatotoxicity, administration of PAP to mice causes liver injury (Song and Chen, 2001). In that study, PAP administration decreased liver GSH concentration without a concomitant increase in glutathione disulfide, and there was only modest protection by ascorbate. Formation of APAP and its conjugates from PAP occurred in both mice and rats (Song and Chen, 2001; Nohynek et al., 2005; Dressler and Appelqvist, 2006), but whether APAP formation contributed to PAP hepatotoxicity was not evaluated.

Because evidence exists for involvement of deacetylation of APAP to PAP in the renal toxicity of APAP, we examined the possibility that deacetylation contributes to the initiation of P450-independent APAP toxicity in HPCs in vitro. Deacetylation of APAP in liver is known to occur, but rapid reacetylation of PAP back to APAP by *N*-acetyltransferases (NATs) in a "futile cycle" occurs at smaller APAP concentrations, so that little PAP accumulates (Nicholls et al., 1997; McConkey et al., 2009). Accordingly, the deacetylation pathway has been viewed as a minor one with little importance in APAP-induced HPC injury. However, its contribution to cytotoxicity in vitro from larger concentrations of APAP may have been underestimated. First, the metabolism of APAP by P450s is limited by inactivation of P450s by reactive NAPQI (Snawder et al., 1994), thereby potentially slowing elimination of APAP and consequently maintaining intracellular concentrations available for deacetylation to PAP. Second, in HPCs compromised by the early-acting P450-dependent mechanism, ATP production is impaired (Andersson et al., 1990; Burcham and Harman, 1991; Harmon et al., 2006). The resultant decrease in ATP availability would be expected to slow NAT-dependent reacetylation of PAP, because formation of acetylCoA as a donor molecule for this reaction requires ATP. The slowing of NAT-mediated reacetylation coupled with the prolonged availability of APAP would favor accumulation of PAP. Indeed, PAP accumulated in cells exposed to a large APAP concentration, and an inhibitor of deacetylation reduced cytotoxicity.

At large APAP concentrations, inhibition of both P450s and deacetylases reduced cytotoxicity more than inhibition of either alone. Accordingly, it may be that PAP produced by deacetylation of APAP at large concentrations in vitro acts on cells stressed by NAPQI to result in pronounced cell death. If PAP contributes to APAP cytotoxicity, it should injure HPCs at concentrations smaller than those caused by APAP, because complete conversion of APAP to PAP is unlikely. Indeed, direct addition of PAP to HPCs was injurious at

concentrations far smaller (approximately 1/40th) than concentrations of APAP needed to cause P450-independent cytotoxicity. When PAP was added to the HPC incubation medium, the medium concentration at which cytotoxicity occurred (approximately $\geq 200 \mu\text{M}$) was greater than the PAP concentration (approximately $8 \mu\text{M}$) measured in the medium of HPCs to which 14 mM APAP had been added. However, in the latter case, the PAP was produced from APAP within the HPCs, in which the PAP concentration would be expected to be far greater than that detected in the culture medium after PAP exited the cells and was diluted by the medium. Finally, because 1-ABT pretreatment failed to affect ALT release induced by exogenously added PAP, injury from PAP under these conditions did not depend on its acetylation to APAP and subsequent bioactivation of APAP by P450s.

Interestingly, cells exposed to large, but not small cytotoxic concentrations of APAP (i.e., $\geq 8 \text{ mM}$) turned brown in color, as did HPCs exposed to cytotoxic concentrations of PAP (i.e., $\geq 250 \mu\text{M}$) (Supplemental Fig. 4). It has been suggested that in solution PAP undergoes oxidation under neutral and alkaline conditions and can polymerize to yield a brown color over time, and others have suspected that this is the likely cause of brown urine and of pigmentation of stored serum from APAP-overdosed patients (Brown et al., 1983; Clark et al., 1986). Our observations suggest that the brown coloration of the cells from exposure to large concentrations of APAP in vitro could be caused by PAP. Together, these results strongly suggest that PAP contributes to the P450-independent cytotoxicity observed at larger APAP concentrations in vitro. It should be noted, however, that BNPP, alone or together with 1-ABT, did not protect HPCs completely from cytotoxicity caused by a large concentration of APAP; this suggests that the deacetylation pathway is not the sole contributor to the P450-independent mode of APAP cytotoxicity in vitro.

Whether the P450-dependent and -independent initiating mechanisms activate similar or different cell death signaling pathways and how PAP contributes to hepatocellular injury remain to be determined. Peyrou et al. (2007a,b) found evidence of endoplasmic reticulum (ER) stress in kidneys of PAP-treated rats. ER stress markers (XBP1, GRP94, GRP78)

were increased in renal tissue. M-Calpain was activated, as was caspase 12, and a calpain inhibitor reduced toxicity (Peyrou et al., 2007b). In HK-2 kidney cells, PAP caused apoptosis and caspase 3 activation (Bai et al., 2012). In Jurkat cells, PAP decreased GSH and caused DNA fragmentation and apoptosis, and phosphorylation of AKT was decreased (Chang et al., 2012). Moreover, 500 mg/kg APAP (i.e., a large dose), but not 250 mg/kg, given to mice caused expression of ER stress markers in liver, even though both of these APAP doses caused liver injury (Uzi et al., 2013). As a possible mechanism of PAP-induced hepatocellular injury, evaluating the contribution of ER stress and also PAP-SG and ROS would be interesting.

The results in isolated HPCs in vitro raise an obvious question as to whether the P450-independent mechanism contributes to APAP-induced liver injury in vivo, given the relatively large concentrations of APAP (i.e., $>5 \text{ mM}$) required to evoke this mechanism. In the classic studies of Jollow et al. (1973), a dose of 375 mg/kg APAP given to mice caused necrosis of centrilobular parenchymal cells that was completely prevented by an inhibitor of P450s. In contrast, a larger dose (750 mg/kg) of APAP caused more extensive necrosis that was reduced, but not eliminated by the P450 inhibitor. This result suggests the possibility of a P450-independent component to hepatotoxicity when large APAP doses are used in mouse studies.

Inasmuch as plasma APAP concentrations in human poisonings do not typically exceed 5 mM, a P450-independent mechanism in APAP-induced liver injury in human cases might not be expected to occur, unless human hepatocytes are more sensitive than mouse hepatocytes to this mechanism.

In conclusion, our data point to at least two mechanisms that contribute to APAP cytotoxicity in vitro: 1) a P450-dependent mechanism that operates at small, cytotoxic APAP concentrations (5 mM or less) and that occurs rapidly and is limited both in degree and duration, and 2) a P450-independent mechanism that predominates at larger concentrations (8 mM and greater), is slower to develop and highly lethal, and might involve deacetylation of APAP to PAP (Fig. 7). Thus, the assumption that P450-mediated formation of NAPQI is solely responsible for hepatocellular death in

Fig. 7. Proposed pathways for APAP-induced HPC injury in vitro. At small but toxic APAP concentrations, conjugation pathways are overwhelmed and the rate of reactive NAPQI formation by P450 increases, leading to HPC stress and early death of some primary mouse HPCs. The deacetylation pathway initially produces minimal PAP because PAP is rapidly reacylated by NATs back to APAP. However, upon exposure to large concentrations of APAP, reacylation is slowed by ATP depletion, enhancing PAP concentration to cytotoxic levels. PAPS, 3'-phosphoadenosine-5'-phosphosulfate; UDP-GA, uridine diphosphate glucuronic acid.

vitro at these larger concentrations is incorrect. These results should provide guidance for choosing APAP concentrations and for interpreting results of studies of APAP cytotoxicity in vitro.

Authorship Contributions

Participated in research design: Miyakawa, Roth, Ganey, Letzig, Lehner, Buchweitz, James, Scott.

Conducted experiments: Miyakawa, Letzig, Lehner, Buchweitz, James, Albee.

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Wrote or contributed to the writing of the manuscript: Miyakawa, Roth, Ganey, Letzig, Lehner, Buchweitz, James, Scott, Albee.

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