Protein Arylation Precedes Acetaminophen Toxicity in a Dynamic Organ Slice Culture of Mouse Kidney

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Acetaminophen (APAP) is a commonly used antipyretic analgesic which may cause hepatotoxicity and nephrotoxicity with overdose in man and laboratory animals. In vivo studies suggest that in situ activation of APAP contributes to the development of nephrotoxicity. Associated with target organ toxicity is selective arylation of proteins, with a 58-kDa acetaminophen binding protein (58-ABP) being the most prominent cytosolic target. In this study a mouse kidney slice model was developed to further evaluate the contribution of in situ activation of APAP to the development of nephrotoxicity and to determine the selectivity of protein arylation. Precision cut kidney slices from male CD-1 mice were incubated with selected concentrations of APAP (0–25 mM) for 2 to 24 hr. APAP caused a dose- and time-dependent decrease in nonprotein sulfhydryls (NPSH), ATP content, and K+ retention. Preceding toxicity was arylation of cytosolic proteins, the most prominent one being the 58-ABP. The association of 58-ABP arylation with APAP toxicity in this mouse kidney slice model is consistent with earlier, in vivo results and demonstrates the importance of in situ activation of APAP for the development of nephrotoxicity. Precision cut renal slices and dynamic organ culture are good models for further mechanistic studies of APAP-induced renal toxicity.

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Acetaminophen (APAP) is a commonly used antipyretic analgesic which may cause liver and kidney damage with overdose in humans (Boyer and Rouff, 1971) and laboratory animals (Boyd and Bereczky, 1966; Mitchell et al., 1973a). Studies using radiolabeled APAP determined that NAPQI arylates cellular macromolecules. This protein arylation is greatest after significant depletion of nonprotein sulfhydryls (NPSH), primarily the nucleophile glutathione (GSH) which is important for APAP detoxification (Mitchell et al., 1973b). Early studies suggested a relationship between protein arylation, NPSH depletion, and toxicity (Jollow et al., 1973; Potter et al., 1974). Studies with affinity-purified antibody which can detect covalently bound APAP have shown that binding is highly selective (Bartolone et al., 1987) and that the selective protein arylation is better associated with hepatic toxicity than is total protein arylation as detected by radioisotopic studies (Beierschmitt et al., 1989). For liver toxicity, a 58-kDa APAP binding protein (58-ABP) is the most prominent target in hepatic cytosol from mice and humans (Bartolone et al., 1988, 1989; Birge et al., 1990). Selective arylation of 58-ABP in kidney has also been associated with APAP-induced nephrotoxicity in CD-1 mice (Emeigh Hart et al., 1991; Hoivik et al., 1995). Recent studies suggest that APAP-glutathione conjugates (APAP-SG) may also contribute to nephrotoxicity in these mice, since probenecid (an inhibitor of organic anion transport) and acivicin (an inhibitor of γ-glutamyl transpeptidase activity) both prevented APAP-induced renal toxicity (Emeigh Hart et al., 1996). The arylation of renal proteins is thought to result from in situ activation of APAP by the kidney since castration of male mice, which diminished renal P450, decreases APAP-induced NPSH depletion, protein arylation, and toxicity (Emeigh Hart et al., 1994). By contrast, administration of testosterone to female mice increases renal CYP2E1 content, bioactivation of APAP, and APAP-induced NPSH depletion, protein arylation, and toxicity (Hoivik et al., 1995). Neither castration nor testosterone treatment had any effect on APAP-induced hepatotoxicity (Emeigh Hart et al., 1994; Hoivik et al., 1995). Taken together, these studies suggest that in situ activation of APAP is contributing to the protein arylation and subsequent development of nephrotoxicity in mice. Indirect support for this may be found in the intrinsic sensitivity to APAP of renal slices from middle-aged rats.
(Tarloff et al., 1990). However, since mice and rats likely differ with respect to the site of activation and nature of the toxic metabolite(s) involved in acetaminophen-induced nephrotoxicity (Emeigh Hart et al., 1991) an in vitro model is needed for mouse kidney to better test the in situ activation hypothesis.

The present studies were undertaken to develop an in vitro model of APAP nephrotoxicity in male CD-1 mice. Precision cut renal tissue slices were chosen because they have several beneficial features when compared to other in vitro techniques. In tissue slices, the cellular architecture is maintained so site-selective injury can be assessed, and since chemical delivery is not dependent on blood flow one can identify cells which are uniquely susceptible to toxicant injury. Tissue slices are used within minutes of isolation so most enzymatic activity is maintained. Moreover, with appropriate conditions, tissue slices can be maintained for 24–36 hr (Tarloff and Goldstein, 1994; Fisher et al., 1995; Parrish et al., 1995). In many cases, the toxic effects in tissue slices mimic those seen in vivo. Thus, tissue slices from guinea pig liver have been utilized to study halothane biotransformation and toxicity (Ghantous et al., 1990) and rat liver slices have been used to examine bromobenzene metabolism, covalent binding, and toxicity (Fisher et al., 1993). Biochemical effects similar to those noted in vivo have been observed with cephaloridine and with mercuric chloride treatments of rat renal slices (Cojocel et al., 1985; Goldstein et al., 1986; Ruegg et al., 1987a), and with cisplatin toxicity in rabbit renal slices (Phelps et al., 1987).

The following studies in mouse renal tissue slices show that APAP causes a dose- and time-dependent toxicity which is associated with NPSH depletion and selective protein arylation, a response similar to what is observed in vivo, and which is consistent with a role for intrarenal activation in APAP-induced nephrotoxicity.

**MATERIALS AND METHODS**

**Chemicals and supplies.** Waymouth’s MB 752/1 powdered medium (without phenol red), L-glutamine, and fetal calf serum were purchased from Gibco Laboratories (Grand Island, NY). Acetaminophen, gentamicin sulfate, amphotericin B, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), goat anti-rabbit IgG peroxidase conjugate, 2-mercaptoethanol, fish gelatin, bromophenol blue, GSH, 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in 10 ml ethanol diluted 1:10 in 0.5 M Tris-HCl, sucrose, 0.01 M 2,5-(50-100 ml/min). Control incubations contained the supplemented media but no APAP.

**Harvesting of tissue slices.** The tissue slices were removed from the incubates at selected times and placed in 1 ml of 10% trichloroacetic acid (TCA) after weighing. The slices were immediately homogenized with a Polytron homogenizer and subsequently flash frozen in liquid nitrogen. Samples were stored at −80°C until analysis which was within 48 hr of harvesting. The slice homogenate was thawed and then centrifuged at 10,000g for 10 min, and the resulting supernatant was used for the determination of ATP, NPSH, and potassium levels. For analysis of covalent binding, a pool of three slices was placed in an Eppendorf tube, flash frozen in liquid nitrogen, and stored at −80°C until analysis. The tissue (approximately 30 mg wet wt) was thawed on ice, suspended in 1.2 ml of STM buffer (0.25 M sucrose, 0.01 M Tris-HCl, and 0.001 M MgCl₂, pH 7.4), and homogenized with a Dounce Teflon homogenizer. The homogenate was centrifuged at 9000g for 20 min and the resulting supernatant was then centrifuged at 105,000g for 1 hr to obtain the cytosolic fraction. Slices were collected from selected incubates and processed for histopathological verification of toxicity as described (Emeigh Hart et al., 1991).

**Determination of nonprotein sulphydryl content.** To assess the NPSH content of slices the method of Sedlak and Lindsay (1968) was employed. Briefly, a 50-μl aliquot of the homogenate supernatant was transferred to a microfuge plate. Ellman’s reagent was added (200 μl of 39.6 mg 5,5-dithiobis-nitrobenzoic acid (DTNB) in 10 ml ethanol diluted 1:10 in 0.5 M Tris-HCl in 10⁻³ M EDTA buffer, pH 8.9). The absorbance at 405 nm is directly proportional to the NPSH content and the concentration was determined by comparison with a standard curve prepared with known concentrations of GSH ranging from 0 to 1.0 mM. Results are expressed as nmol NPSH/mg wet weight.

**Western blot analysis of APAP protein arylation.** Cytosolic proteins were separated by SDS–polyacrylamide gel electrophoresis according to molecular weight as described by Laemmli (1970). Protein concentrations were determined by the method of Lowry et al. (1951). Western blot analy-
Effect of APAP on Nonprotein Sulfhydryl Content of Renal Slices

Conjugation with the nucleophile GSH is a major detoxification pathway for the reactive intermediate of APAP (Mitchell et al. 1973b). With biotransformation of APAP to NAPQI, NPSH levels decline and thus are an indirect indicator of APAP activation (Mitchell et al., 1973b). In control slices NPSH content was maintained throughout the 24 hr (Fig. 1). Exposure to 1 mM APAP, the lowest concentration tested, decreased renal slice NPSH by 44–55% throughout the time course of the study (2–24 hr). Increased concentrations of APAP resulted in greater depletion of NPSH in a dose-dependent manner. In general, at concentrations of 5 mM and above the NPSH decline appeared to increase with time, resulting in near complete depletion with 25 mM APAP after 24 hr of continuous exposure.

APAP Protein Arylation in Renal Tissue Slices

Figure 2 shows the 58-kDa region from Western blots of cytosol from renal slices. APAP binding was most prominent.
FIG. 3. Effect of APAP exposure on ATP content in renal slices from male CD-1 mice. Mouse renal tissue slices were isolated as described under Materials and Methods. They were incubated for selected intervals in Waymouth's medium containing the indicated concentrations of APAP [0 mM (○), 1 mM (■), 5 mM (△), 10 mM (▼), and 25 mM (●)]. At the indicated times the incubations were terminated and tissue ATP content was determined. The data are expressed as mean ± SE (n = 3 replicate cultures). An asterisk indicates the treatment is significantly different from the control at the indicated time.

Effect of APAP exposure on renal ATP content. APAP at 1 mM caused a 7–12% loss of potassium over the 24-hr incubation (Fig. 4). Greater potassium loss occurred with increased APAP exposure, with losses of 22, 40, and 65% attained after 24 hr incubation with 5, 10, or 25 mM APAP, respectively (Fig. 4). Histopathological examination of selected slices exposed to APAP (10 mM, 8–12 hr) revealed APAP-induced injury to the renal proximal tubules (not shown) consistent with what we have previously observed in vivo (Emeigh Hart et al., 1991).

DISCUSSION

Incubation of precision cut kidney slices from male CD-1 mice with APAP resulted in a dose- and time-dependent decrease in ATP and potassium. ATP was decreased earlier and after lower concentrations of APAP than was potassium and this differential sensitivity of these two indicators of slice viability is consistent with previous findings using liver slices and other toxicants (Fisher et al., 1995a). In general significant decreases in potassium were only detected after treatment with the higher concentrations of APAP and only with the longest exposure periods. By contrast, significant ATP depletion was observed as early as 2 hr after 25 mM APAP and at 12 hr after as little as 1 mM. This decrease in ATP content prior to significant loss of potassium may reflect prelethal events such as inhibition of mitochondrial function which has been documented in APAP-induced liver toxicity (Esterline and Ji, 1986; Meyers et al., 1988; Burcham and...
Harman, 1991). By contrast, loss of potassium from slices more likely reflects a loss of membrane integrity that would occur in association with cell death (Fisher et al., 1995a). With in vivo exposure to toxic doses of APAP, NPSH depletion and protein arylation in target tissues generally precede biochemical evidence of cell injury (Hoivik et al., 1995). Consistent with the in vivo findings, exposure of kidney slices to 1 to 25 mM APAP resulted in significant depletion (48 and 57%) of NPSH at 2 hr, the earliest time point investigated. For 1, 5, and 10 mM APAP this is well in advance of any significant ATP depletion, although there was 21% depletion after 25 mM APAP. As indicated above, significant potassium loss required much longer exposure to APAP. The temporal relationship for protein arylation was also consistent with in vivo models of hepatic and renal APAP toxicity. Thus, the most prominent target was a 58-kDa cytosolic protein, e.g., 58-ABP (Bartolone et al., 1988; Emeigh Hart et al., 1991; Hoivik et al., 1995), and binding was clearly demonstrable well in advance of ATP or potassium depletion. However, by contrast with what is generally observed in vivo, for each concentration tested, protein arylation increased with time through 12 hr, although for the higher APAP concentrations binding diminished when the exposure was increased from 12 to 24 hr, the times when potassium depletion was greatest. In vivo, binding generally reaches a maximum by 2 to 4 hr after dosing and declines thereafter (Bartolone et al., 1987). These differences likely reflect the different nature of APAP exposure between in vivo and cultured slice models. In the former, APAP is rapidly cleared from blood and tissues (Fischer et al., 1985; Anker and Smilkstein, 1994). In culture, however, the APAP remained throughout the duration of incubation allowing for continued APAP activation which could account for the continued increase in binding.

These studies are the first to utilize precision cut mouse kidney slices for the evaluation of APAP’s nephrotoxicity. The data suggest that APAP toxicity in mouse kidney slices reflects the response seen in vivo (Emeigh Hart et al., 1991; Hoivik et al., 1995). Consistent with a role for the electrophile NAPQI in toxicity, NPSH depletion and 58-ABP arylation preceded ATP depletion and potassium loss in a dose- and time-dependent fashion. This study documents in situ activation of APAP within renal slices supporting the earlier proposals from in vivo studies in mice that APAP activation within the kidney contributes to the renal covalent binding and toxicity. Dynamic mouse kidney slice cultures will be a useful in vitro model for future mechanistic studies of APAP-induced nephrotoxicity.

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