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SELECTIVE PROTEIN ARYLATION BY ACETAMINOPHEN AND 2,6-DIMETHYLACETAMINOPHEN IN CULTURED HEPATOCYTES FROM PHENOBARBITAL-INDUCED AND UNINDUCED MICE

RELATIONSHIP TO CYTOTOXICITY*

RAYMOND B. BIRGE,† JOHN B. BARTOLONE,† DENIS J. MCCANN,‡ JAMES B. MANGOLD,‡ STEVEN D. COHEN§ and EDWARD A. KHAIRALLAH†‖

Departments of † Molecular and Cell Biology, ‡ Medicinal Chemistry and Pharmacognosy, and § Pharmacology and Toxicology, The University of Connecticut, Storrs, CT 06269, U.S.A.

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Abstract—To evaluate the mechanistic importance of covalent binding in acetaminophen (APAP)-induced hepatotoxicity, we compared the effects of 2,6-dimethylacetaminophen (2,6-DMA) to those of APAP in primary cultures of mouse hepatocytes. Immunochemical analysis of electrophoretically separated proteins has shown that the majority of covalent binding after a cytotoxic dose of APAP occurs on two major bands of 44 and 58 kD (Bartolone *et al.*, *Biochem Pharmacol* **36**: 1193–1196, 1987). At equimolar concentrations, 2,6-DMA bound proteins only 15% as extensively as did APAP and was not cytotoxic in hepatocytes from uninduced mice. However, when the hepatocytes were obtained from phenobarbital-induced mice, APAP administration resulted in increased protein arylation and a more rapid onset of cytotoxicity. Furthermore, in the cells from phenobarbital-induced mice, 2,6-DMA not only resulted in increased binding but also in overt cytotoxicity. Since our affinity-purified anti-APAP antibody did not cross-react with 2,6-DMA, a new antibody specific for 2,6-DMA was prepared and, after affinity purification, was used to detect 2,6-DMA protein adducts by Western blotting. Results indicated that, in hepatocytes from both phenobarbital-induced and non-induced mice, the binding of 2,6-DMA was also highly selective with the most prominent target being the 58 kD cytosolic protein. However, by contrast to APAP, only minimal binding to the 44 kD protein was detected after 2,6-DMA treatment. Although several additional protein adducts were increased in treated cells from phenobarbital-induced mice, the 58 kD protein was clearly the most prominently arylated target associated with both APAP and 2,6-DMA cytotoxicity. These data suggest that both the specificity of covalent binding as well as the extent of binding to the major targets may play an important role in the ensuing toxicity.

Acetaminophen (APAP) is a widely used analgesic which is considered safe at therapeutic levels. However, at excessive doses, severe centrilobular necrosis can result which is often fatal to laboratory animals and humans [1–3]. Hepatotoxicity is thought to result from the cytochrome P-450 mediated oxidation of APAP to *N*-acetyl-*p*-benzoquinone imine (NAPQI) [4, 5]. Although the exact mechanisms by which NAPQI exerts it cytotoxic effects are not understood completely, good correlations are noted between the extent of covalent binding and the severity of hepatic necrosis [2, 6]. Thus, inhibition of NAPQI generation by the administration of cytochrome P-450 inhibitors [7] or by scavenging the reactive electrophile by compounds such as *N*-acetylcysteine [8, 9] greatly diminishes the cellular damage caused by APAP, while inducing the synthesis of cytochrome P-450 isozymes that activate APAP [7] or depleting the intracellular concentrations of GSH [1, 10] increases the extent of covalent binding and exacerbates the hepatotoxicity. These data suggest that the mechanism of toxicity may reside, at least in part, in the covalent binding of reactive metabolites to critical cellular macromolecules. However, several investigators have questioned the mechanistic importance of protein arylation to the ensuing cell damage [11–13]. Indeed, based upon studies which demonstrate that the toxicity of APAP can be prevented with anti-oxidants [11, 12] and flavones [13] without a reduction in the extent of covalent binding, some investigators have suggested that the hepatotoxicity may result from the oxidative properties of NAPQI [14]. Furthermore, several structurally related compounds, such as the 2′- and 3′-hydroxy isomers of APAP [15 16], or 2,6-dimethyl acetaminophen (2,6-DMA) [17], have been reported to bind covalently to proteins without eliciting hepatotoxicity. Similar results have been observed with bromobenzene whereby several cytochrome P-450 mediated metabolites can covalently bind proteins but only some appear to be associated with the toxicity [18]. Thus, *p*-bromophenol, a major bromobenzene metabolite, binds to approximately 60% the extent of a similar dose of bromobenzene, yet only

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‖ Address correspondence to: Dr Edward A. Khairallah, Department of Molecular and Cell Biology, U-125, The University of Connecticut, 75 North Eagleville Road, Storrs, CT 06269.

the latter induces hepatotoxicity [19]. All such challenges to the covalent binding hypothesis have either moderated toxicity without altering binding or have demonstrated binding in the absence of toxicity. However, no studies have demonstrated toxicity in the absence of binding. Therefore, binding may still be critical to hepatotoxicity, and it is likely that the toxicological significance of metabolite covalent binding to proteins may lie in the specificity and extent of arylation of the target molecules.

To better understand the toxicological significance of APAP covalent binding, our laboratory had developed an immunochemical assay to detect electrophoretically resolved APAP-bound proteins. The results indicated that about 85% of the covalent binding detected both in liver after *in vivo* exposure and in cultured hepatocytes after incubation with hepatotoxic concentrations of APAP occurred on two major protein bands of molecular weight 44 and 58 kD [17, 20–22]. This high degree of selectivity suggests that these arylated proteins may be important in the APAP-mediated toxicity. Recently, we have reported that, although radiolabeled 2,6-DMA covalently binds to proteins to a much lesser degree than APAP, it does not result in cytotoxicity in cultured hepatocytes from non-induced mice [17]. By contrast, it has been reported that 2,6-DMA can cause toxicity in cells from phenobarbital-induced rats [23]. In this study, we report the production of an affinity-purified antibody which detects 2,6-DMA-bound proteins and its use, in parallel with the anti-APAP antibody [20, 21] to compare the selective binding of APAP and 2,6-DMA and to assess the relationship of the specificity of protein binding to the resulting hepatotoxicity in cultured hepatocytes. These studies demonstrate that the cytotoxicities of both APAP and 2,6-DMA correlate with the increased arylation of but a few cellular proteins.

MATERIALS AND METHODS

Materials. Collagenase CLS-II and peroxidase-linked goat anti-rabbit IgG were obtained from Organon Teknika (BCA Cappel, Westchester, PA). Nitrocellulose membranes (0.45 μm) were purchased from Schleicher & Schuell (Keene, NH). Epoxy-activated Sepharose 6B was obtained from Pharmacia (Piscataway, NJ). Electrophoretic grade Tris–HCl, acrylamide, *N,N'*-methylene bis-acrylamide, glycine, and sodium dodecyl sulfate (SDS) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). 2,6-Dimethylbenzoquinone was purchased from Aldrich (Milwaukee, WI). Both 2,6-DMA and [^{3}H]-2,6-DMA were synthesized as previously described by Fernando *et al.* [24] and modified by our laboratory [17]. *N*-Acetyl-*p*-benzoquinone imine (NAPQI) and *N*-acetyl-2,6-dimethylbenzoquinone imine (2,6-demethyl NAPQI) were synthesized by the silver oxide procedure originally described by Streeter *et al.* [25]. Uniformly labeled [^{3}H]APAP and ^{125}I-conjugated goat anti-rabbit IgG were obtained from Dupont New England Nuclear (Boston, MA). All other reagents were purchased from the Sigma Chemical Co. (St Louis, MO).

Preparation of hepatocytes. Male C57-Bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Phenobarbital-induced mice received 1 mg/ml of sodium phenobarbital (pH 7.5) in their drinking water for at least 5 days prior to use and 24 hr before hepatocyte isolation the mice received an intraperitoneal injection (40 mg/kg) of sodium phenobarbital (40 mg/ml in 0.9% NaCl) [26]. Hepatocytes were isolated from 3 to 4-month-old mice by a two-step collagenase perfusion method [27] as described and modified in our laboratory [9]. The isolated hepatocytes were washed and plated on 35 mm Falcon tissue culture dishes (0.75×10^6 cells/plate) in Eagle's Minimum Essential Medium (MEM) supplemented with nonessential amino acids, 10% fetal bovine serum, 10 μM dexamethasone, 2 μM insulin, 15 mM nicotinamide, 50 μg/ml gentimycin, 200 μg/ml streptomycin, and 200 units/ml penicillin. The cells were allowed to adhere for approximately 18 hr prior to the initiation of experiments in fresh medium lacking nicotinamide. APAP or 2,6-DMA was added directly to this fresh medium to provide a final concentration of 10 mM.

Conjugation of hapten and immunization. The procedured utilized to elicit antibodies against protein-bound 2,6-DMA were similar to those previously reported by our laboratory for the production of antibodies against protein bound-APAP [20, 21]. Briefly, the immunogen was constructed by the mixed anhydride method [28] in which 2,6-DMA was conjugated to keyhole limpet hemocyanin (KLH). Since 2,6-DMA does not contain a carboxyl group, 40 mmol of 2,6-DMA (containing 0.12 mCi/mmol [^{3}H]-2,6-DMA as a tracer) was initially reacted with 40 mmol diazotized *p*-aminobenzoic acid (PABA) (12.6 ml final volume) [29]. After derivatization, the product was lyophilized to complete dryness and dissoved in 1.0 ml of dimethylformamide at 10° containing 35 μl of isobutyl chloroformate to render the carboxyl groups of PABA reactive towards amino groups on KLH [28]. The addition of a solution containing 1 mg/ml KLH in 50 mM $NaHCO_3$ buffer (pH 9.6) resulted in the production of a stable hapten–protein conjugate. After gentle shaking for 16 hr at 4°, the KLH–PABA–2,6-DMA conjugate was dialyzed for 48 hr against several changes of phosphate-buffered saline (PBS), pH 7.4. Isotopic analysis indicated that the final product contained 30.9 mol of 2,6-DMA per subunit mol of KLH.

Male New Zealand white rabbits (3- to 4-months-old) were immunized by multiple subcutaneous injections of approximately 400 μg of the KLH–PABA–2,6-DMA immunogen emulsified with Freund's complete adjuvant (800 μl final volume). Booster injections of the immunogen (200 μg) were administered in Freund's incomplete adjuvant at approximately 1-month intervals, and the antisera was collected and stored frozen in aliquots at −70° until further use.

Characterization of anti-2,6-DMA antibodies by ELISA. Reactivity of polyclonal antibodies directed towards 2,6-DMA was assayed by ELISA [30]. Briefly, wells on polystyrene microtiter plates were coated for 1 hr at 37° with either 100 ng of BSA (in 0.05 M carbonate–bicarbonate buffer, pH 9.6) or with BSA which had been derivatized previously

with 2,6-dimethyl NAPQI to yield an adduct containing 0.71 mol 2,6-DMA/mol BSA. Since the immobilized antigen contained neither *p*-aminobenzoic acid nor KLH, the assay is specific for anti-2,6-DMA antibodies. Microtiter plates were washed five times with PBS containing 0.05% Tween-20 (PBS-Tween) and incubated with 50 μl of serially diluted antisera (1:4 to 1:1024) for 3 hr at 37°. Subsequently, the wells were washed again and incubated with 50 μl of horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG (1:200) for 1 hr. After a final wash, the anti-2,6-DMA antibodies were detected by the addition of the peroxidase substrate [2,2′-azino-di-(3-ethylbenzthaline sulfonic acid] prepared in 50 mM citrate buffer (pH 4.0) containing 0.12% hydrogen peroxide. Peroxidase activity was monitored at 405 nm on a computer-interfaced Artek model 210 plate reader. Anti-2,6-DMA activity was detected at sera dilutions up to 1:1024 (data not shown). For competitive ELISA, the microtiter plates were coated with 500 μg/well of 2,6-DMA derivatized BSA or BSA alone and, after washing, 50 μl of 0.12 to 500 μg/ml of 2,6-DMA, APAP, 3-hydroxy-APAP, 2-hydroxy-APAP, or 2,6-dimethylbenzoquinone in PBS-Tween was added to separate wells in duplicate. Immediately following, 50 μl of affinity purified antibody (see below) was added at a final dilution of 1:32 and incubated for 3 hr at 37°, and peroxidase activity was monitored as described.

Anti-2,6-DMA antibody affinity purification. The crude IgG fraction was initially precipated by the addition of 40% ammonium sulfate (final concentration) to the immune sera [20, 21]. After reconstitution with PBS to the original volume, the samples were dialyzed against PBS, and 1.0-ml aliquots were loaded onto a 3.0-ml column of prewashed epoxy-activated Sepharose 6B to which 2,6-DMA was covalently coupled [21]. The non-absorbed fraction (monitored at 280 nm) was eluted at 25° with PBS, and when no further protein was detected, the affinity-bound fraction was eluted off the column by the addition of 5.0 ml of a 5 mg/ml solution of 2,6-DMA in PBS. The bound 2,6-DMA was removed from the anti-2,6-DMA antibodies by extensive dialysis against PBS at 4°. Affinity purification increased the specific activity of the antisera 110-fold over the unfractionated antisera (data not shown).

Immunochemical detection of APAP and 2,6-DMA bound proteins in mouse hepatocytes. To evaluate the specificity of the covalent binding, medium containing either 10 mM APAP or 2,6-DMA was added to hepatocyte cultures obtained from either phenobarbital-induced or uninduced mice. At the indicated exposure times, the cells were harvested and homogenized in a hypotonic buffer (10 mM Tris–HCl, 15 mM KCl, and 5 mM EDTA, pH 7.4) and then separated into particulate and supernatant fractions by centrifugation at 105,000 g for 1 hr. Subsequently, protein aliquots from each fraction were resolved electrophoretically on 10% SDS–polyacrylamide gels as described by Laemmli [31] and electroblotted onto nitrocellulose membranes (12 × 14 cm) for 6 hr at 75 V in buffer containing 25 mM Tris–HCl, 192 mM glycine, and 20% methanol. The membranes were then blocked overnight at 4° in a 10 mM Tris–HCl solution in 0.9% NaCl (TBS) containing 1% BSA [32]. After blocking, the membranes were incubated for 4 hr at 25° with either 1:400 of the affinity-purified anti-APAP antibody or 1:200 of the affinity-purified anti-2,6-DMA antibody. After rinsing, the targeted proteins were detected following incubation with ^{125}I-conjugated goat anti-rabbit IgG for 1 hr and visualized on Kodak Xar-5 X-ray film after autoradiography for 24–48 hr at −70°.

Isotopic determination of APAP and 2,6-DMA covalent binding. The extent of covalent binding of APAP and 2,6-DMA was also determined isotopically by incubating the hepatocyte cultures with a 10 mM concentration of either [^{3}H]-APAP (0.09 mCi/mmol) or [^{3}H]-2,6-DMA (0.45 mCi/mmol). After 4 hr the cells were harvested and fractionated as described above except that the fractionated proteins were precipitated by the addition of perchloric acid (1 N final concentration) and centrifuged at 2000 *g* for 5 min. The acid-precipitable protein pellets were washed three times with ice-cold 80% methanol containing excess unlabeled APAP or 2,6-DMA, and the radioactivity was determined as previously described [2]. The amount of labeling was corrected for non-specific binding or trapping of the isotopes by subtracting the amount of isotope detected in aliquots of untreated homogenates that were incubated with radiolabeled APAP or 2,6-DMA for 5 min at 4° and then fractionated as described.

Analytical procedures. Cell toxicity was assessed from the percentage of total intracellular glutamate oxaloacetate transaminase (GOT) activity that was released into the medium [33]. Protein concentrations were determined by the method of Lowry *et al.* [34] using bovine serum albumin as a standard. Statistical differences between treated samples were analyzed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity of APAP and 2,6-DMA. The toxicities of APAP and 2,6-DMA were compared in cultured hepatocytes obtained from phenobarbital-induced or uninduced mice by monitoring the extent of GOT activity released into the culture medium. In both induced and uninduced cells the cytotoxicity of APAP increased steadily as a function of exposure time and by 8 hr the amount of GOT released was significantly greater in the cultures obtained from phenobarbital-induced mice (Fig. 1A). By contrast, GOT leakage after 2,6-DMA exposure was significant only in cells from the induced mice (Fig. 1B). The enzyme leakage noted after 2,6-DMA addition to hepatocytes from uninduced mice was not different from that observed in control cells from either induced or uninduced mice and did not exceed 15% through 24 hr of culture (data not shown).

Isotopic analysis of covalent binding of APAP and 2,6-DMA. To compare the extent of the covalent binding of the two drugs, the hepatocytes from induced or uninduced mice were exposed to a 10 mM concentratiaon of either [^{3}H]-APAP (0.09 mCi/

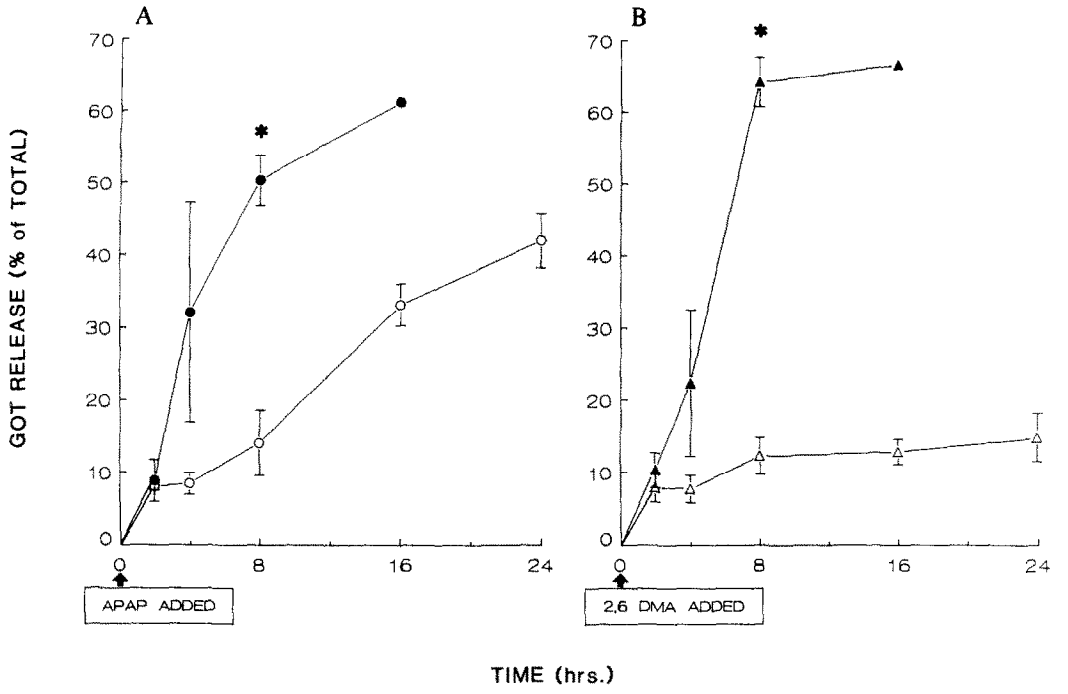

Fig. 1. Effects of APAP and 2,6-DMA on cytotoxicity. Cultured hepatocytes from phenobarbital-induced (●, ▲) or uninduced (○, △) mice were exposed to 10 mM APAP (A) or 2,6-DMA (B) continually for the indicated times. The cytotoxic effects were monitored as the percentage of GOT activity released into the extracellular medium after the exposure. Values are the means ± SE of at least three different experiments except at 16 hr with phenobarbital which represent replicate analysis. Key: (*) $P < 0.05$ versus corresponding time points for hepatocytes from uninduced mice. GOT leakage in controls from either induced or uninduced mice did not exceed 15% over 24 hr (data not shown). Each plate contained approximately 1 mg protein and exhibited GOT activity capable of converting 0.3 to 0.4 μmol of α-ketoglutarate to glutamate per min at 25°, which was measured by the rate of formation of 2,4-dinitrophenylhydrazone at 546 nm [33].

Table 1. Assessment of covalent binding to proteins by APAP and 2,6-DMA in cultured mouse hepatocytes

	Covalent binding (nmol/mg protein)	
	−Phenobarbital	+Phenobarbital
(A) 2,6-DMA		
Cytosol	0.14 ± 0.03	0.71 ± 0.10*
Particulate	0.19 ± 0.03	0.32 ± 0.18
(B) APAP		
Cytosol	0.96 ± 0.18	1.51 ± 0.17†
Particulate	1.56 ± 0.20	1.73 ± 0.28

Covalent binding of radiolabeled APAP or 2,6-DMA was determined in cultured hepatocytes from phenobarbital-induced or uninduced mice after a 4-hr exposure. Hepatocytes were homogenized in a hypotonic buffer (10 mM Tris–HCl, 15 mM KCl, and 5 mM EDTA, pH 7.4), and particulate and cytosolic fractions were obtained by centrifugation at 105,000 *g*. Values are expressed as nanomoles of drug bound per milligram of cellular protein and are the means ± SE of at least three independent experiments.

* Statistically significant at the $P < 0.01$ level.

† Statistically significant at the $P < 0.05$ level.

mmol) or [^{3}H]-2,6-DMA (0.45 mCi/mmol) for 4 hr and then fractionated into particulate and cytosolic fractions. Total covalent binding of APAP in cells from the uninduced mice was 7-fold greater than that of 2,6-DMA (Table 1). Phenobarbital pretreatment significantly increased the extent of cytosolic [^{3}H]-APAP binding from 0.96 nmol/mg protein to 1.51 nmol/mg protein and of [^{3}H]-2,6-DMA binding from 0.14 nmol/mg protein to 0.71 nmol/mg protein. No statistically significant changes were noted in particulate binding when cells from the induced mice were compared to fractions from uninduced mice.

Characterization of antibody epitope specificity. The epitope specificity of the affinity-purified anti-2,6-DMA antibody was analyzed by competitive ELISA utilizing several structurally related compounds (Fig. 2). Inhibition of antibody binding by the free hapten is interpreted to indicate antibody cross-reactivity. All four analogues tested diminished antibody binding at concentrations of 10 ng/well or greater. However, the antibody exhibited an approximately 1000 times greater specificity towards 2,6-DMA (Fig. 2). The fact that APAP (which contains an *N*-acetyl moiety but no methyl substitution in the 2′ and 6′ positions) as well as 2,6-dimethylbenzoquinone (which contins 2′- and 6′-methyl substitution but no *N*-acetyl moiety) were much less reactive with this anti-2,6-DMA antibody suggests that both methyl substitution and the *N*-acetyl moiety together comprise a critical epitope center necessary for antibody recognition. It should be noted that, although the antibody exhibited slight cross-reactivity toward APAP, 3-hydroxy-APAP and 2-hydroxy-APAP, no protein adducts were detected

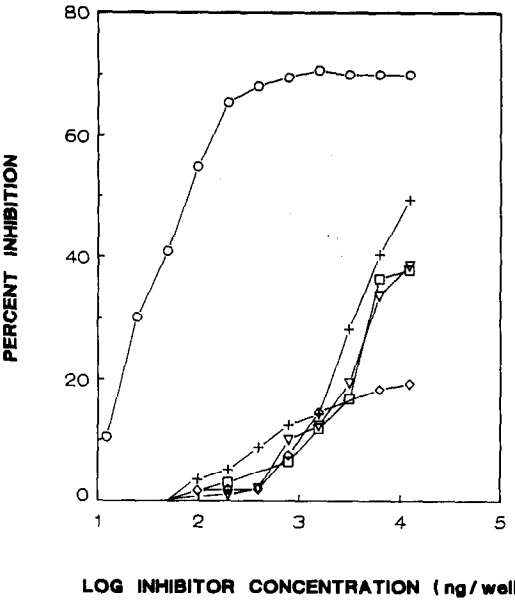

Fig. 2. Characterization of the substrate specificity of the affinity-purified anti-2,6-DMA antibody. The cross-reactivity of the antibody with free 2,6-DMA (○), APAP (+), 3-OH-APAP (□), 2-OH-APAP (△), and 2,6-dimethylbenzoquinone (◊) was determined by a competitive ELISA using HRP peroxidase-conjugated goat anti-rabbit IgG as described under Materials and Methods. Results are expressed as percent inhibition of peroxidase activity compared to wells in which no inhibitors were added.

immunochemically by the anti-2,6-DMA antibody after the addition of a 10 mM concentration of each of these compounds in the hepatocyte cultures (data not shown). This concentration was chosen because it readily yields detectable APAP protein adducts with a similarily produced anti-APAP antibody [17].

Electrophoretic separation of hepatocyte proteins from phenobarbital-induced and uninduced mice. To compare the specificity of APAP and 2,6-DMA protein adducts, cultured hepatocytes were initially obtained from either phenobarbital-induced or uninduced mice prior to drug exposure. Upon removal of the medium, the cells were fractionated to yield particulate and cytosolic fractions. When equal amounts of protein from each cell fraction were electrophoretically resolved, the protein patterns revealed by the Coomassie blue staining indicated that several proteins were induced by phenobarbital. Most noteworthy were increases in the protein content of two bands of 52 and 54 kD in the particulate fraction and of a 27 kD band in the cytosol (Fig. 3). However, no further differences in the Coomassie protein patterns were noted if APAP of 2,6-DMA were added for 2, 4, or 8 hr to cultures from either phenobarbital-induced or uninduced mice (data not shown).

Immunochemical detection of the proteins targeted by APAP. Consistent with our previous observations [20, 21], the immunochemical analysis revealed that the binding of APAP in the cells from the uninduced mice (Fig. 4, upper panel (-PB)] was selective and mainly localized on a 58 kD cytosolic protein (arrow 1) and a 44 kD protein (arrow 2) present in both the particulate (P) and cytosolic (S) fractions. In addition a 52 kD protein (arrow 3) was detected in the particulate fractions especially at 4 and 8 hr. The immunoblots also provided qualitative confirmation of the quantitative isotopic data (Table 1) that covalent binding in the cytosolic fractions was greater in cells from phenobarbital-induced mice (+PB). Most prominent was the phenobarbital-dependent increase in the binding to the cytosolic 58 kD protein. Also noteworthy were the increases in cytosolic APAP adducts of 27, 33, 44, and 130 kD in hepatocytes from the induced mice.

Even though the isotopic binding of APAP in the particulate fractions was not significantly different in cells from induced and uninduced mice at 4 hr (Table 1), the immunochemical analysis showed that the qualitative pattern of covalent binding in this fraction changes upon phenobarbital-induction. The most striking difference was the relative decrease in binding to the 44 kD protein in the particulate fraction of cells from the induced mice not only at 4 hr (time at which the isotopic analysis was conducted) but also at 2 and 8 hr. In addition, the immunoblots revealed that in these cells APAP binding to the 52 kD protein appeared to increase and by 8 hr binding to a 54 kD protein was also noted. The 52 and 54 kD particulate targets may represent the proteins that were increased by phenobarbital-induction (Fig. 3).

Immunochemical detection of the proteins targeted by 2,6-DMA. The lower panels of Fig. 4 demonstrate that the binding of 2,6-DMA to proteins, although on a relative scale appeared less prominent than that with APAP, was also highly selective. In cytosol of cells from the uninduced mice only the 58 kD target was prominent at 8 hr. Although minor bands of 27 and 130 kD were also faintly detectable in the cytosol, no selective 2,6-DMA targets were detected in the particulate fraction of the cells from the uninduced mice.

The immunochemical analysis of the proteins targeted by 2,6-DMA also provided qualitative confirmation of the isotopic data (Table 1) that hepatocytes from phenobarbital-induced mice exhibited greater 2,6-DMA binding than cells from uninduced mice. Similar to APAP, the most notable increase in 2,6-DMA binding was to the 58 kD protein which became prominent as early as 2 hr. Cytosolic proteins of 33 and 130 kD also became noticeable expecially at 4 hr. In addition, among several other minor adducts, the arylation of a 44 kD cytosolic protein was faintly detectable but only in the cells from the phenobarbital-induced mice. In the particulate fraction of these cells the arylation of the 52 kD at 4 and 8 hr was most prominent; no particulate 44 kD protein adduct of 2,6-DMA was detected in these studies.

DISCUSSION

Although the exact biochemical events associated with APAP-induced hepatic necrosis are not fully understood, no one has yet been able to demonstrate APAP toxicity in the absence of any covalent binding; the covalent binding of the electrophilic intermediate, NAPQI, to cellular proteins has found widespread acceptance as relevant to the ensuing toxicity [2]. We have demonstrated recently that

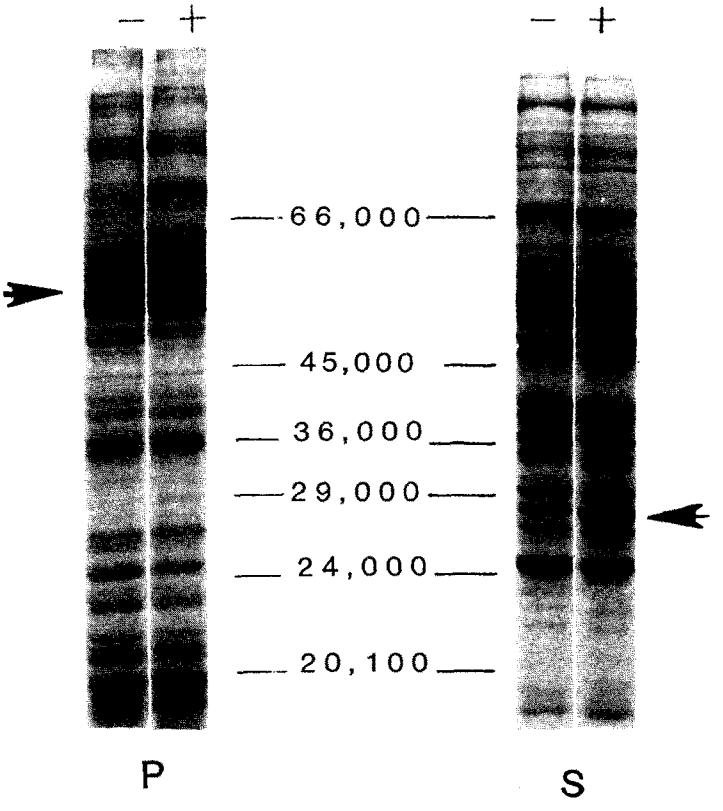

Fig. 3. SDS–PAGE and Coomassie brilliant blue staining of proteins from cultured hepatocytes from phenobarbital-induced and uninduced mice. Proteins from cultured control hepatocytes obtained from phenobarbital-induced (+) or uninduced (−) mice were resolved on SDS–PAGE (10% gel) as described under Materials and Methods. The electrophoretic mobility of each molecular weight standard is noted. (P) refers to the particulate or 105,000 *g* pellet and (S) refers to the cytosolic fraction contained in the 105,000 *g* supernatant fraction. Arrows indicate the protein bands that were prominently increased in cells from phenobarbital-induced mice. No further differences in proteins were noted after 2, 4, or 8 hr of drug treatment (data not shown).

such binding is not random, but highly selective [17, 20, 21, 35]. Since phenobarbital administration not only exacerbates APAP toxicity but also increases the extent of binding to cellular proteins [7], we have utilized the immunochemical approach to demonstrate the specificity of the increased protein arylation associated with the enhanced APAP toxicity in cultured cells from phenobarbital-induced mice. Findings indicate that the enhanced toxicity observed in hepatocytes from phenobarbital-induced mice results in an increase in the covalent binding to the cytosolic 44 and 58 kD proteins and a decrease in the arylation of the particulate 44 kD protein. In addition, proteins of approximately 27, 33, 52–54 and 130 kD, which were only minimally arylated in the cells from the uninduced mice, were also prominently targeted by APAP after phenobarbital induction. Hence, an analysis of both the qualitative specificity as well as the relative extent of binding to the arylated protein targets may be essential not only for a clearer understanding of the increased sensitivity to APAP seen in the hepatocytes from the phenobarbital-induced mice but also of the toxicological significance of covalent binding in general.

In an effort to better evaluate the significance of the selective APAP binding, we have reported recently that, at equimolar concentrations to APAP, 2,6-DMA can bind to cellular proteins, albeit to a lower extent than APAP, without resulting in hepatotoxicity [17]. By contrast, Porubek *et al.* [23] have reported that 2,6-DMA can be cytotoxic when added to hepatocyte suspensions prepared from phenobarbital-induced Sprague–Dawley rats. Our present data demonstrate that primary cultures from phenobarbital-induced C57/Bl 6 mice, in contrast to cells from uninduced mice, were also susceptible to damage by 2,6-DMA. Generation of an affinity-purified antibody which detects 2,6-DMA covalently bound to proteins permitted comparison of the selectivity and relative extent of the covalent binding of 2,6-DMA to that of APAP itself.

Analysis of the Western blot profiles revealed that the few proteins targetted in 2,6-DMA-treated cells were similar in molecular weight to those arylated by APAP, suggesting that the targets of these two drugs are likely to be the same proteins. In the cells from the uninduced mice, where 2,6-DMA did not result in hepatocyte damage, relatively limited arylation of the cytosolic 58 kD protein was observed. By contrast, in the 2,6-DMA-treated hepatocytes from phenobarbital-induced animals in which cytotoxicity was noted, greater arylation of the cytosolic 58 kD protein binding was evident. In addition, protein adducts of 33 and 130 kD were also prominent

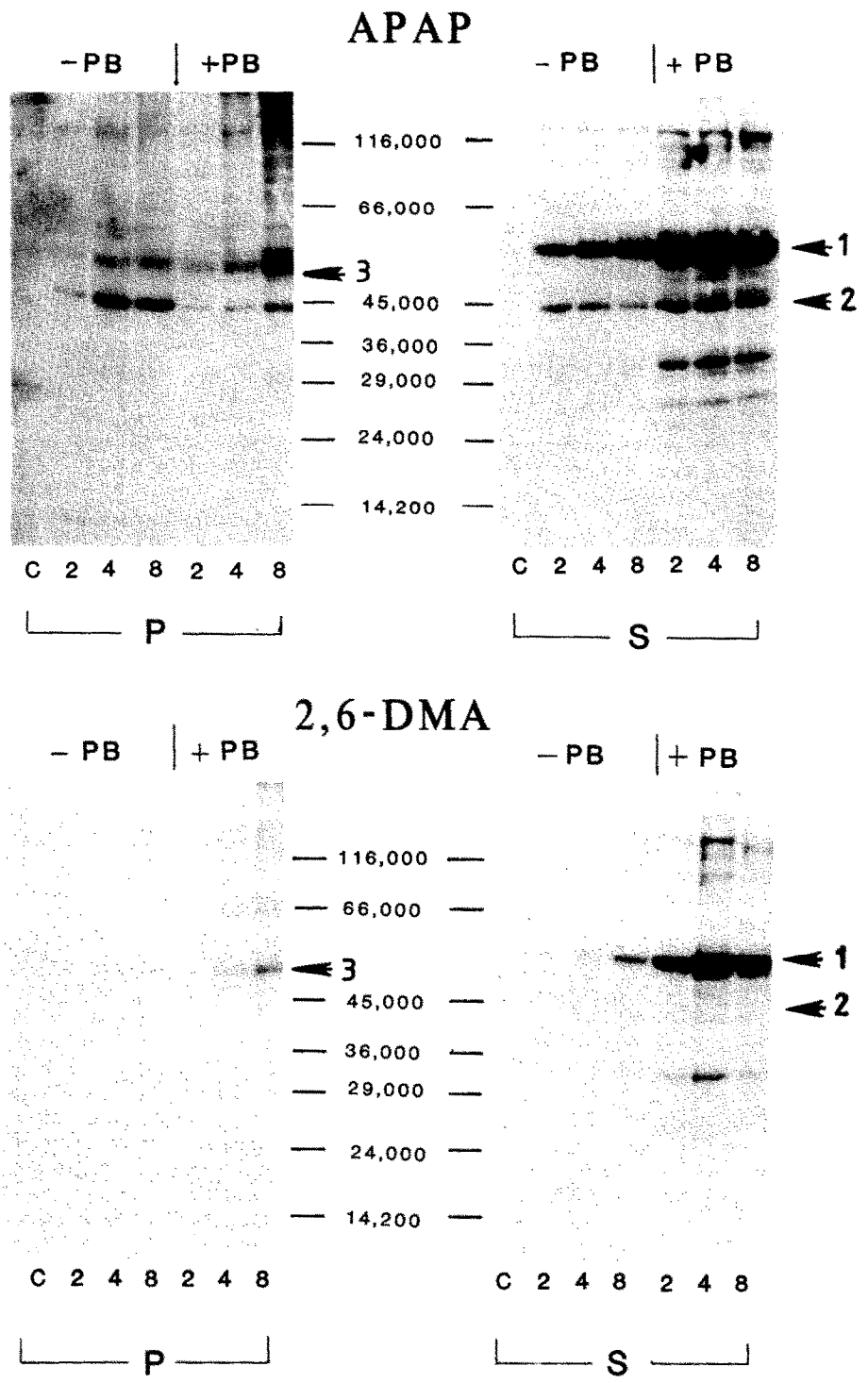

Fig. 4. Western blots of proteins from cultured hepatocytes treated with APAP or 2,6-DMA. Proteins from cultured hepatocytes were obtained from phenobarbital-induced (+PB) or uninduced (−PB) mice and were resolved on SDS–PAGE and transferred to nitrocellulose membranes for Western blotting. In the upper panels, proteins are from hepatocytes treated with APAP for 2, 4, and 8 hr and incubated with an anti-APAP antibody [21]. In the lower panels, proteins are from hepatocytes treated with 2,6-DMA for the indicated times and incubated with an anti-2,6-DMA antibody. In all experiments (P) refers to the particulate or 105,000 *g* pellet and (S) refers to the cytosolic fraction. Visualization of covalent binding was achieved by incubating the nitrocellulose membranes with ^{125}I-conjugated goat anti-rabbit IgG as described under Materials and Methods. The electrophoretic mobility of each molecular weight standard is noted. Arrows (denoted 1, 2, and 3) indicate the localization of the 58, 44, and 52 kD protein bands respectively.

in cytosolic extracts of these induced cells. However, to our surprise, arylation of the 44 kD protein was not detectable in the particulate fractions of either group of cells and was only minimally detectable after 4 hr in the cytosolic fraction in the cells from the phenobarbital-induced mice. The significance of the differences in the selective protein arylation by APAP and 2,6-DMA is not yet clear.

Comparisons of the proteins targetted by 2,6-DMA to those arylated by APAP enables the discrimination of at least three general classes of proteins that become arylated in culture: (1) proteins, exemplified by the 44 kD target, that are arylated extensively by APAP but minimally or not at all by 2,6-DMA; (2) proteins, like the 27, 33, 52–54, and 130 kD targets, whose arylation by both APAP and 2,6-DMA is minimal in conditions that do not result in cell damage but which become much more prominent when cytotoxicity is observed especially in cells from phenobarbital-induced mice; and (3) the cytosolic 58 kD protein, which is clearly the most extensively targeted protein band by either APAP or 2,6-DMA in hepatocytes from either induced or uninduced mice.

The earliest target of APAP covalent binding observed in hepatocytes from uninduced mice is the particulate 44 kD protein [22, 36]. However, in cells from phenobarbital-induced animals, APAP arylation of this protein was decreased concomitant with an increase in the appearance in the cytosol of an APAP-bound protein of similar molecular weight. This may reflect release of the arylated particulate protein into the cytosol and is consistent with our previous observations that covalent binding to this protein in cytosol-free microsomal extracts incubated with APAP and an NADPH-generating system can result in the dislodgement of the arylated 44 kD protein from a particulate to a soluble form [35]. Hence, increased targetting of the particulate form on this protein in cells from phenobarbital-induced mice, may cause its release into the cytosol.

The differences in the relative extent of arylation of the 44 kD protein by APAP and 2,6-DMA may be explained by either of two mechanisms. The first argues that the differences may reside in the oxidative properties of the respective quinone-imines [37]. We reported recently that, in cell culture, APAP exhibits both oxidative and arylative properties, whereas 2,6-DMA possesses only the electrophilic arylative properties [17]. Thus, the oxidative effects of APAP metabolites may destabilize the 44 kD protein or its membrane environment and enhance its likelihood for covalent binding. An alternative explanation argues that, *in situ*, the 44 kD protein may be localized in close proximity to a cytochrome P-450 isozyme that activates APAP but may be inaccessible by either compartmentation or steric hindrance from the site of 2,6-dimethyl NAPQI generation. These two possibilities are currently under investigation. Nevertheless, the fact that 2,6-DMA did not target this protein with great avidity in culture, even when cytotoxicity was demonstrated, indicates that the arylation of the 44 kD protein is not likely to be critical for the onset of 2,6-DMA-mediated hepatotoxicity, even though its importance in APAP toxicity cannot be ruled out.

The second group of protein targets appears to be prominently arylated in cultured cells that eventually exhibit hepatotoxicity. Three of them (the 27, 33, and 54 kD proteins) became targetted only in hepatocytes from phenobarbital-induced C57 Bl/6 mice where both APAP and 2,6-DMA exhibited cytotoxic effects, while the other two proteins of 52 and 130 kD proteins were also minimally arylated by cytotoxic concentrations of APAP in cells from uninduced mice. It is clear from the Coomassie staining that the cellular contents of the 52 and 54 kD particulate proteins, as well as the cytosolic 27 kD protein, were increased by phenobarbital pretreatment. The 52–54 kD proteins likely represent cytochrome P-450 isozymes which are known to be induced by phenobarbital [38–41]. Thus, targetting of these proteins may be related to their role in metabolizing APAP and 2,6-DMA to their respective reactive metabolites. The significance of the targetting of the 27 and 33 kD proteins by both APAP and 2,6-DMA is still unclear.

The cytosolic 58 kD protein is the most intensively arylated target of either APAP or 2,6-DMA. A low degree of arylation of this protein, as exemplified by exposure to sub-toxic doses of APAP *in vivo* [20, 21] or in culture [22] or to 2,6-DMA in cells from uninduced mice, appears to be tolerated and does not result in hepatotoxicity. However, the extent of covalent binding to this protein was greatly increased under all conditions that resulted in cell damage. These observations can be interpreted to suggest that the 58 kD protein may be either a protective or a critical protein. In the first case, it can be argued that the 58 kD protein represents an electrophile scavenging protein which may serve a *protective* function to decrease the intracellular concentration of the reactive quinone-imines. This notion is consistent with our recent observations that the native 58 kD protein is highly reactive towards *N*-ethylmaleimide at pH 7.4 [21], suggesting that the 58 kD protein is very nucleophilic at physiological pH and hence would be a likely target of attack by reactive electrophiles. Thus, at below threshold concentrations of APAP and 2,6-DMA, this protein may, by virtue of its reactive thiols, be well suited to bind the quinone-immines and thereby prevent further interaction of the metabolite with other targets. Hepatotoxicity would result when critical proteins become arylated. The existence of such a cyto-protective electrophile binding protein may be analogous to the role of metallothionein in the binding and detoxification of heavy metals [42–44]. However, an alternative explanation of the data is that the 58 kD protein may itself represent a *critical* enzyme or structural protein that is relatively abundant within the cell. Limited arylation of such a protein may not be sufficiently disruptive of cellular function to result in irreversible damage. However, once a critical amount of such a protein is altered by arylation, its functional reserve may be become overwhelmed and cell damage would ensue. A further assessment of these two alternatives is warranted.

The results presented above demonstrate that the marked similarities in the arylation patterns of 2,6-DMA and APAP suggest that these compounds may be exerting their cytotoxic effects by a common

biochemical mechanism. Both APAP and 2,6-DMA do not bind randomly, but very selectively. However, not all covalent binding is of apparently equal significance. Since the 44 kD protein was targeted extensively by APAP but only minimally by 2,6-DMA, then the arylation of this protein may not be involved in 2,6-DMA-related cell damage. By contrast, the extensive arylation of the 58 kD protein by both APAP and 2,6-DMA was most prominently associated with the cytotoxicity. This protein may serve as a protective "quinone-binding" protein that initially diverts reactive electrophiles away from other more critical targets or it may itself be a critical target that, once overwhelmed by extensive binding, would disrupt cell function. These data suggest that the elucidation of the qualitative nature of the targets as well as the extent of their arylation will be essential for a clearer understanding of the role that covalent binding may play in cell toxicity.

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