Biochemical Pharmacology, Vol. 40, No. 3, pp. 573–579, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

IMMUNOCHEMICAL QUANTITATION OF 3-(CYSTEIN-*S*-YL)ACETAMINOPHEN PROTEIN ADDUCTS IN SUBCELLULAR LIVER FRACTIONS FOLLOWING A HEPATOTOXIC DOSE OF ACETAMINOPHEN

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(*Received* 13 *July* 1989; *accepted* 19 *December* 1989)

Abstract—The hepatotoxicity of acetaminophen correlates with the formation of 3-(cystein-*S*-yl)acetaminophen protein adducts. Using a sensitive and specific immunochemical assay, we quantitated the formation of these protein adducts in liver fractions and serum after administration of a hepatotoxic dose of acetaminophen (400 mg/kg) to B6C3F1 mice. Adducts in the cytosolic fraction increased to 3.6 nmol/mg protein at 2 hr and then decreased to 1.1 nmol/mg protein by 8 hr. Concomitant with the decrease in adducts in the cytosol, 3-(cystein-*S*-yl)acetaminophen protein adducts appeared in serum and their levels paralleled increases in serum alanine aminotransferase. Microsomal protein adducts peaked at 1 hr (0.7 nmol/mg protein) and subsequently decreased to 0.2 nmol/mg at 8 hr. The 4000 *g* pellet (nuclei, plasma membranes, and cell debris) had the highest level of adducts (3.5 nmol/mg protein), which remained constant from 1 to 8 hr. Evaluation of fractions purified from a 960 *g* pellet indicated that the highest concentration of 3-(cystein-*S*-yl)acetaminophen protein adducts was located in plasma membranes and mitochondria; peak levels were 10.3 and 5.1 nmol/mg respectively. 3-(Cystein-*S*-yl)acetaminophen protein adducts were detected in nuclei only after enzymatic hydrolysis of the proteins. The localization of high levels of 3-(cystein-*S*-yl)acetaminophen protein adducts in plasma membranes and mitochondria may play a critical role in acetaminophen toxicity.

Acetaminophen is a safe analgesic; however, in overdose it produces a fulminating hepatic necrosis [1–4]. The toxicity of acetaminophen is caused by a reactive metabolite, which is believed to be the two-electron oxidation product of acetaminophen, *N*-acetyl-*p*-benzoquinone imine (NAPQI;§ [5]). Following low doses of acetaminophen, NAPQI is efficiently detoxified by glutathione forming the conjugate, 3-(glutathion-*S*-yl)acetaminophen [6, 7]. In acetaminophen overdose, or other circumstances that lead to depletion of glutathione, the reactive metabolite covalently binds to cellular proteins. The relative amount of this covalent binding correlates with the severity of the toxicity. Available evidence suggests that covalent binding of acetaminophen to protein in animals is a reaction between NAPQI and cysteinyl sulfhydryl groups on protein to produce the corresponding 3-(cystein-*S*-yl)acetaminophen protein adduct [8, 9].

Since covalent binding is a reliable biomarker for production of the reactive metabolite, we developed a sensitive and specific immunoassay to quantitate specific 3-(cystein-*S*-yl)acetaminophen adducts [10] and characterized the epitope recognized by the antisera [11]. Using this immunoassay, we defined the temporal relationship between acetaminophen hepatotoxicity in mice and the formation of the specific 3-(cystein-*S*-yl)acetaminophen adduct in the 10,000 *g* liver supernatant fraction and in serum proteins after hepatotoxic doses of acetaminophen [12].

The present study correlates the distribution of covalent adducts in several major liver fractions to the release of 3-(cystein-*S*-yl)acetaminophen protein adducts into the serum and their temporal relationship to acetaminophen toxicity. Using B6C3F1 male mice, we quantitated 3-(cystein-*S*-yl)acetaminophen protein adducts at 1, 2, 4, 6 and 8 hr after a 400 mg/kg dose of acetaminophen in the 4000 *g* pellet (plasma membranes, nuclei, and cell debris), 15,000 *g* pellet (mitochondria), 100,000 *g* pellet (microsomes), and 100,000 *g* supernatant (cytosol) liver fractions. Serum alanine aminotransferase (ALT) levels were used as a correlate of hepatotoxicity. The finding of relatively high levels of persistent adducts in the 4000 *g* pellet prompted further investigation of the covalent binding of the components comprising this fraction. Using differential and gradient centrifugation, plasma membrane, nuclear, and mitochondrial fractions were prepared from mouse livers removed at 0.5, 1, 2, and 4 hr after administration of 400 mg/kg acetaminophen. The levels of 3-(cystein-*S*-yl)acetaminophen protein adducts in each fraction were quantitated using a specific and sensitive particle concentration fluorescence immunoassay.

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§ Abbreviations: NAPQI, *N*-acetyl-*p*-benzoquinone imine; PCFIA, particle concentration fluorescence immunoassay; A-B ELISA, avidin biotin-amplified enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; and ALT, alanine aminotransferase.

MATERIALS AND METHODS

Reagents. Acetaminophen and protease type XIV were purchased from the Sigma Chemical Co. (St. Louis, MO). Dithiothreitol and sodium azide were procured from the Eastman Kodak Co. (Rochester, NY). Spectrapor dialysis tubing with a molecular weight cutoff of 2000 daltons was obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA). Fetal calf serum was a product of Gibco (Grand Island, NY). Fluorescein conjugated mouse anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Animals. Six-week-old male B6C3F1 mice having an average weight of 20 g were obtained from the NCTR specific pathogen-free breeding colony. Animals were housed four to a cage in clear plastic cages with hardwood bedding, and provided type 5010 M laboratory chow (Ralston-Purina, St. Louis, MO). The animals were maintained on a 12-hr light–dark cycle with temperature and humidity controlled. Food was removed from the animals at 5:00 p.m. prior to the morning of dosing (8:00 a.m.). Mice were dosed with 400 mg acetaminophen/kg body weight by intraperitoneal injection (i.p.) of 0.27 mL/10 g body weight of a 40° dosing solution containing 15 mg acetaminophen/mL of pyrogen-free saline (Travenol Laboratories Inc., Deerfield, IL). For the initial time–course study, fasted 6- to 8-week-old, male B6C3F1 mice were dosed i.p. with 400 mg/kg acetaminophen and killed at 1, 2, 4, 6, and 8 hr, and 3-(cystein-*S*-yl)acetaminophen protein adduct levels were determined in crude liver fractions. For the second time course, mice were killed at 0.5, 1.0, 2.0, and 4.0 hr after dosing, and 3-(cystein-*S*-yl)acetaminophen protein adducts in plasma membranes, nuclei and mitochondria from pooled livers (four/time point) were determined. Saline-treated animals killed at 4 hr were used as controls.

Sample preparation. Blood samples were obtained from the retro-orbital plexus of mice anesthetized with CO_2. The blood was allowed to clot at room temperature, and serum was separated using serum separator (Sure Sep II, General Diagnostics, Morris Plains, NJ) and stored at −60° prior to analysis. Serum ALT levels were performed with a Baker Encore autoanalyzer and Baker CentrifiChem ALT optimized reagents (Baker Instrument Co., Allentown, PA) according to the method described by Bergmeyer *et al.* [13].

Liver fractionation. Mouse liver 4000 *g*, 15,000 *g*, and 100,000 *g* pellets and 100,000 *g* supernatant fraction were prepared essentially as described previously [14, 15]. Liver tissue was homogenized with 3 vol. of isotonic phosphate-buffered saline (0.01 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl; PBS) using a motor-driven glass–Teflon homogenizer. The homogenate was centrifuged at 4000 *g* for 10 min and maintained at 4° during all subsequent sample manipulations. The 4000 *g* supernatant fraction was centrifuged for 20 min at 15,000 *g* forming a pellet enriched in mitochondria. The post-mitochondrial fraction was centrifuged at 100,000 *g* for 1 hr. The 4000 *g*, 15,000 *g*, and 100,000 *g* pellets were resuspended in an appropriate volume of PBS using a Dounce homogenizer and recentrifuged to wash the pellets. The 4000 *g*, 15,000 *g*, and 100,000 *g* pellets as well as the 100,000 *g* supernatant fraction and serum were dialyzed against five 1-L changes of PBS prior to immunochemical quantitation of adducts. The mean percentages of total protein recovered in each liver fraction were: 4000 *g* pellet, 52.7%; 15,000 *g* pellet, 6.9%; 100,000 *g* pellet, 5.7%; and 100,000 *g* supernatant fraction, 34.7%. In acetaminophen-treated animals (400 mg/kg, 4 hr), the mean percentages of protein recovered in each liver fraction were: 4000 *g* pellet, 49.7%; 15,000 *g* pellet, 6.0%; 100,000 *g* pellet, 5.7%; and 100,000 *g* supernatant fraction, 38.6%.

Mitochondria, plasma membranes, and nuclei were isolated using the method by Fleischer and Kervina [16]. Samples were dialyzed against five 1-L changes of PBS and stored at −70° until assayed.

Immunochemical quantitation of 3-(cystein-S-yl)acetaminophen adducts. Acetaminophen protein adducts were measured using a competitive particle concentration fluorescence immunoassay (PCFIA). Like the previously described avidin biotin-amplified enzyme-linked immunosorbent assay (A-B ELISA), the PCFIA assay is based on rabbit antisera raised against 3-(cystein-*S*-yl)acetaminophen conjugated to keyhole-limpet hemocyanin [10]. This antisera (previous designation Rab-541) has been characterized extensively and has great specificity for acetaminophen covalently bound at carbon 3 to the sulfhydryl of cysteine residues on protein [11]. The PCFIA is essentially analogous to the A-B ELISA, but the assay format differs in several ways: (1) the immobilized assay antigen (NAPQI modified metallothionein) is covalently bound to polystyrene assay particles instead of adsorbed to the wells of polystyrene microtiter plates; (2) unbound antibody is removed by vacuum filtration instead of washing; and (3) the bound antibody is detected with a fluorescein conjugated second antibody instead of a biotinylated second antibody followed by avidin-biotin-horseradish peroxidase mediated conversion of a substrate to a chromophore. In either assay, the presence of acetaminophen-protein adducts in a sample or standard competitively inhibits the binding of primary antibody to immobilized antigen and thus inhibits the signal generated by the fluorescein- or biotin-conjugated second antibody. The PCFIA and the A-B ELISA have similar sensitivity and recognize the same epitope, but the PCFIA has several advantages including a covalently bound immobilized assay antigen, shorter incubation times, and microprocessor controlled automation.

The assay was performed as described previously [10–12] with modifications to adapt the assay to the PCFIA format. A limiting amount of specific 3-(cystein-*S*-yl)acetaminophen adduct rabbit IgG antibody was incubated with either 3-(*N*-acetyl-L-cystein-*S*-yl)acetaminophen standard or an unknown (mouse liver fraction or mouse serum), for 45 min at 37°. All dilutions were prepared in 16 mM phosphate buffer, pH 7.4, 140 mM NaCl, 5 mM KCl (isotonic buffered saline, IBS) with 4% (v/v) fetal bovine serum (FBS). Equal volumes (25 μL) of inhibitor–antibody mixture and 0.127% (w/v) solution of assay particles (NAPQI modified metallothionein covalently bound to 0.89 μm amino-polystyrene beads)

were added to the wells of specially designed assay plates (Pandex Division of the Baxter Health Care Corp., Mundelein, IL). Plates were incubated at 37° for 30 min and then washed three times with 0.05% (v/v) NP-40 in IBS (wash buffer). Subsequently, 25 μL of fluorescein-labeled mouse anti-rabbit IgG diluted 1:250 was added to each assay well and incubated at room temperature for 30 min. Finally, the plate was washed five times with wash buffer, and the remaining fluorescence was quantitated at 485 nm excitation, 535 nm emission using a Fluorescence Concentration Analyzer (Baxter Health Care Corp.).

The protein concentration of dialyzed samples was determined by a Coomassie blue dye-binding assay (Bio-Rad, Richmond, CA; [17]) using bovine plasma albumin as a standard. Unknowns were diluted to a final concentration of approximately 4 μg/well and assayed in duplicate. Inhibition by unknown samples was compared with a standard curve prepared using radiolabeled [^{3}H]-3-(*N*-acetyl-L-cystein-*S*-yl)-acetaminophen. The radiolabel was used to verify the concentration of bound acetaminophen in the standard. The values obtained were corrected for differences in the relative inhibitory potency of 3-(*N*-acetyl-L-cystein-*S*-yl)acetaminophen and 3-(cystein-*S*-yl)acetaminophen protein adducts (120 fmol/well and 2300 fmol/well respectively; [11]), and expressed as nanomoles of adducts per milligram protein. Previously, certain NAPQI modified protein fractions bound antibody more efficiently after hydrolysis with pronase [11]. The decision whether or not to treat individual fractions with pronase was based on parallel evaluations of pronase-treated and untreated pooled liver fractions. Protein (1 mg/mL) was digested overnight at 37° with pronase (0.1 mg/mL) in 10 mM sodium phosphate (pH 7.4). Before quantitating the sample in the immunochemical assay, a final concentration of 10 mM EDTA was added to inhibit further protein hydrolysis. Pronase treatment did not interfere with the detection of identically treated 3-(*N*-acetyl-L-cystein-*S*-yl)acetaminophen standard.

Statistics. A two-tailed Student's *t*-test was used to make the comparisons between experimental and control levels of alanine aminotransferase and 3-(cystein-*S*-yl)acetaminophen protein adducts. Differences were considered statistically significant when $P \leq 0.05$.

RESULTS

Previously, an A-B ELISA [10] was used to quantitate 3-(cystein-*S*-yl)acetaminophen adducts in 10,000 *g* supernatant fraction [12]. The assay system was characterized extensively [11] and recognized 3-(cystein-*S*-yl)acetaminophen adducts over 300-fold more efficiently than acetaminophen. 3-(Cystein-*S*-yl)acetaminophen protein adducts in this experiment were quantitated using an analogous PCFIA. The PCFIA and the A-B ELISA recognized the same epitope as demonstrated by similar relative inhibitory potencies for 3-(*N*-acetyl-L-cystein-*S*-yl)acetaminophen, synthetic NAPQI derivatized glutathione *S*-transferase, and acetaminophen when assayed in both assay systems (data not shown).

Time course for hepatotoxicity and protein adducts. The temporal relationship between hepatotoxicity and 3-(cystein-*S*-yl)acetaminophen protein adducts in the hepatic 4000 *g* pellet, 15,000 *g* pellet, 100,000 *g* pellet, 100,000 *g* supernatant fraction and serum was evaluated in fasted B6C3F1 male mice dosed with 400 mg acetaminophen/kg body weight. Adducts in liver fractions and the hepatotoxicity were evaluated at 1, 2, 4, 6, and 8 hr after dosing.

The major portion of the 3-(cystein-*S*-yl)acetaminophen protein adducts was contained in the 4000 *g* pellet and 100,000 *g* supernatant fraction. 3-(Cystein-*S*-yl)acetaminophen protein adducts in the cytosolic fraction (100,000 *g* supernatant) peaked at 2 hr (3.6 nmol/mg) after a hepatotoxic dose of acetaminophen and declined to a value of 1.1 nmol/mg protein at 8 hr (Fig. 1A). This decline in 3-(cystein-*S*-yl)acetaminophen protein adducts correlated temporally with the rise in protein adducts detected in the serum (Fig. 1E). Peak levels of 3-(cystein-*S*-yl)acetaminophen protein adducts in the liver coincided with the initial detection of protein adducts in the serum. The 3-(cystein-*S*-yl)acetaminophen adducts detected in the 4000 *g* pellet maintained a sustained plateau of approximately 3.5 nmol/mg protein 1–8 hr after dosing. Levels of 3-(cystein-*S*-yl)acetaminophen protein adducts in the 4000 *g* pellet were variable within each group of animals, suggesting either large variability in individual animals for this fraction or variability in sample preparation (Fig. 1B). In the 15,000 *g* pellet, immunochemically detectable 3-(cystein-*S*-yl)acetaminophen protein adducts were less than 0.5 nmol/mg protein and significantly ($P \leq 0.05$) different from saline controls only at the 1- and 4-hr time points (Fig. 1C). The 100,000 *g* pellet did not contain significant levels of 3-(cystein-*S*-yl)acetaminophen protein adducts.

In our previous work which examined microsomes treated with synthetic NAPQI, it was determined that pronase hydrolysis was necessary for maximal detection of 3-(cystein-*S*-yl)acetaminophen adducts [11]. Therefore, to assess the effect of hydrolysis on the efficiency of antibody recognition, *in vivo* liver fractions were pooled and treated with pronase. The inhibitory potencies of cytosol, 4000 *g* pellet, and serum were unaffected by pronase treatment in the immunoassay; therefore, the data presented in Figs. 1, A, B and E represent non-pronase-treated samples. The 15,000 *g* pellet (mitochondrial fraction) and the 100,000 *g* pellet (microsomal fraction) required prior pronase hydrolysis for full antibidy recognition (Fig. 1, C and D). Pronase treatment dramatically increased recognition of adducts in these samples. 3-(Cystein-*S*-yl)acetaminophen protein adduct levels in the pronase-treated 15,000 *g* pellet remained constant at approximately 2 nmol/mg protein throughout the time course. Peak 3-(cystein-*S*-yl)acetaminophen protein adduct levels in the pronase-treated 100,000 *g* pellet (Fig. 1D) were 0.7 nmol/mg at 1 hr after dosing.

ALT levels have been shown to be a good indicator of acetaminophen hepatotoxicity [18, 19]. The serum ALT levels were significantly ($P \leq 0.05$) different from controls at 2 hr and linearly increased from 2 to 8 hr after dosing (Fig. 1F). Serum 3-(cystein-*S*-yl)acetaminophen protein adducts were also initially

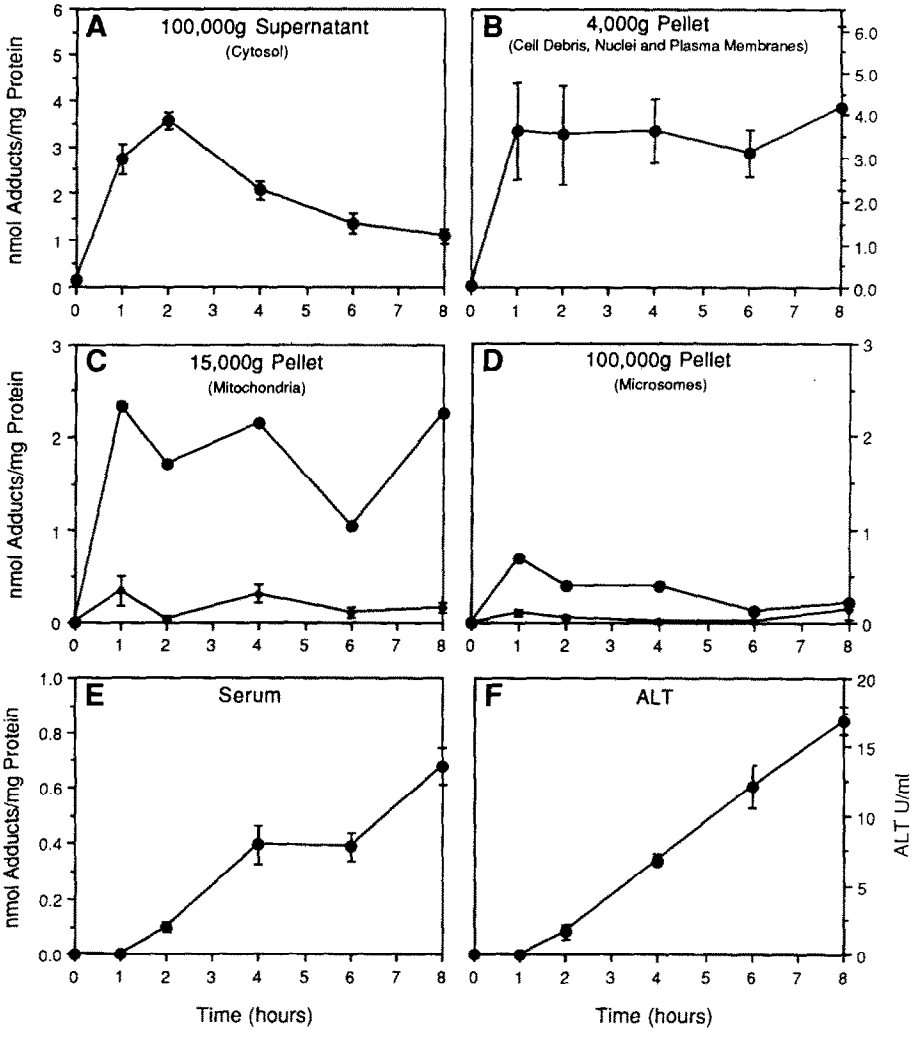

Fig. 1. Time course for 3-(cystein-*S*-yl)acetaminophen adduct formation in liver fractions and serum: Fasted, 6- to 8-week-old, male B6C3F1 mice were dosed i.p. with 400 mg/kg acetaminophen and killed at 1, 2, 4, 6, and 8 hr. Controls were saline-treated mice killed 4 hr after dosing. 3-(Cystein-*S*-yl)acetaminophen protein adducts were quantitated in: non-hydrolyzed 100,000 *g* supernatant containing the cytosolic fraction (A); non-hydrolyzed 4000 *g* containing the plasma membranes, nuclei, and cell debris (B); non-hydrolyzed (◆) and hydrolyzed (●) 15,000 *g* pellet containing the mitochondrial fraction (C); non-hydrolyzed (■) and hydrolyzed (●) 100,000 *g* pellet containing the microsomal fraction (D); and serum (E). The increase in serum ALT levels is shown in panel F. The data are means ± SE (N = 5 mice). The replicates for 100,000 *g* pellet samples gave very little deviation; therefore, the standard error bars are indistinguishable from the closed square symbol (D). The upper curves in C and D are pooled (N = 5) hydrolyzed samples.

detected at 2 hr after dosing (0.1 nmol/mg protein) and increased to 0.7 nmol/mg by 8 hr (Fig. 1E).

Purified hepatic fractions. The sustained levels of 3-(cystein-*S*-yl)acetaminophen protein adducts in the 4000 *g* pellet and the possible importance of covalent binding in this crude fraction indicated that we should further separate the components comprising the 4000 *g* pellet. Fasted male B6C3F1 mice were dosed with 400 mg/kg acetaminophen and killed at 0.5, 1.0, 2.0, and 4.0 hr after dosing. Plasma membranes, mitochondria, and nuclei were purified from a 960 *g* pellet according to the method of Fleischer and Kervina [16] using differential and density centrifugation. ALT levels were used as an indicator of hepatotoxicity and were significantly ($P \leq 0.05$) different from control values 2 hr after dosing. As shown in Fig. 2, pronase-treated nuclei contained 3-(cystein-*S*-yl)acetaminophen protein adducts at levels of 1.9, 1.5, and 2.9 nmol/mg protein at 1, 2, and 4 hr after dosing. Hydrolyzed mitochondria contained 5.1 nmol adduct/mg protein 30 min after dosing which is prior to overt toxicity as determined by the increase in serum ALT levels (Fig. 2). Values for the mitochondria remained constant at approximately 5 nmol adduct/mg protein from 30 min to 2 hr after dosing and then decreased to 2.7 nmol adduct/mg protein at 4 hr after dosing. 3-(Cystein-*S*-yl)-acetaminophen protein adducts in the plasma membranes were also detected at 0.5 hr after dosing and increased significantly ($P \leq 0.05$) to 8.2 nmol adduct/mg protein by 1 hr after dosing. Peak 3-(cystein-*S*-yl)acetaminophen protein adduct levels (10.3 nmol/

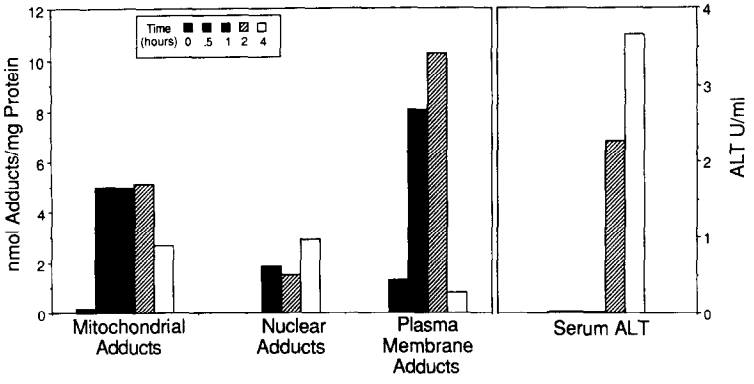

Fig. 2. Quantitation of 3-(cystein-*S*-yl)acetaminophen adducts as a function of time in plasma membranes, nuclei and mitochondria from liver following a hepatotoxic dose of acetaminophen. Fasted, 6- to 8-week-old, male B6C3F1 mice were dosed with 400 mg/kg acetaminophen and killed at 0.5, 1.0, 2.0, and 4.0 hr after dosing. Plasma membranes, mitochondria, and nuclei were purified from a 960 *g* pellet according to a method by Fleischer and Kervina [16]. The data points represent adduct levels from four pooled livers. 3-(Cystein-*S*-yl)acetaminophen protein adducts could be detected in nuclei and mitochondria only after pronase treatment. Pronase treatment did not increase the detection of 3-(cystein-*S*-yl)acetaminophen protein adducts in plasma membranes. Serum ALT levels are shown in the frame on the right-hand side of the figure.

mg protein) were detected in the plasma membranes 2 hr following a hepatotoxic dose of acetaminophen. 3-(Cystein-*S*-yl)acetaminophen protein adducts in the plasma membranes decreased to 0.8 nmol/mg protein by 4 hr.

DISCUSSION

The specific covalent binding of the reactive metabolite of acetaminophen, NAPQI, to proteins involves covalent binding to sulfhydryl groups on protein [9]. The 3-(cystein-*S*-yl)acetaminophen protein adduct constituted over 70% of the covalent adducts observed in mouse liver proteins. Therefore, we developed an immunoassay specific for 3-(cystein-*S*-yl)acetaminophen protein adducts [10]. The epitope recognized by this antiserum was characterized previously by evaluating over thirty structurally related compounds [11]. The major structural requirement was the cysteinyl sulfhydryl group on protein covalently bound ortho to the hydroxyl group of acetaminophen. We previously examined the relationship between the development of acetaminophen hepatotoxicity and the binding to cysteinyl sulfhydryl groups on proteins [12] and found that the occurrence of 3-(cystein-*S*-yl)acetaminophen protein adducts in the liver 10,000 *g* supernatant proteins precedes the release of ALT into the serum. Covalently bound 3-(cystein-*S*-yl)acetaminophen protein adducts were observed in serum from mice dosed with acetaminophen. The appearance of these 3-(cystein-*S*-yl)acetaminophen protein adducts in serum correlated with the release of ALT from the hepatocytes in a dose-dependent and temporal fashion. The disappearance of liver adducts in the 100,000 *g* supernatant proteins temporally correlated with the appearance of protein adducts detected in the serum. In our current studies we have examined the temporal relationship between 3-(cystein-*S*-yl)acetaminophen protein adducts in various liver fractions to the occurrence of protein adducts in the serum and to the development of hepatotoxicity as measured by the release of ALT from the liver. Since we found large quantities of persistent 3-(cystein-*S*-yl)acetaminophen protein adducts in the 4000 *g* pellet, we further investigated the subcellular components comprising this fraction.

Confirming our previous data [12], the 3-(cystein-*S*-yl)acetaminophen protein adducts detected in serum temporally correlated with the release of ALT from the liver (Fig. 1). Both the ALT levels and the 3-(cystein-*S*-yl)acetaminophen protein adducts detected in the serum were significantly ($P \leq 0.05$) different from controls 2 hr after dosing. The observed 3-(cystein-*S*-yl)acetaminophen protein adducts temporally correlated with the loss of protein adducts from the 100,000 *g* supernatant fraction. Collectively, the observed decrease of 3-(cystein-*S*-yl)acetaminophen protein adducts in the 100,000 *g* supernatant fraction and the temporal relationship between the appearance of ALT in the serum and the parallel appearance of 3-(cystein-*S*-yl)acetaminophen protein adducts in the serum suggests that the 3-(cystein-*S*-yl)acetaminophen protein adducts are released from the 100,000 *g* supernatant (cytosolic) liver fraction. ALT in the hepatic cytosol is released into the serum following damage or destruction to the hepatocytes or increased permeability of the plasma membrane [20, 21]. Presumably, the release of 3-(cystein-*S*-yl)acetaminophen protein adducts from the cytosol of hepatocytes into serum is similar. The subcellular distribution of [^{3}H]acetaminophen protein adducts following a 375 mg/kg dose of acetaminophen to mice was reported by Jollow *et al.* [22] using a radiolabeled assay for acetaminophen bound to protein. In their study, the highest levels occurred in the soluble (cytosolic) and microsomal fractions with comparatively little in the nuclear and cell debris fractions and moderate amounts in the mitochondrial fraction. In the present study, the majority of 3-(cystein-*S*-yl)acetaminophen protein adducts was located in the cytosolic and 4000 *g* pellet fractions. The 4000 *g* pellet contains nuclei, plasma membranes, and cell

debris along with some mitochondria. The 4000 *g* pellet contained substantial and persistent amounts of 3-(cystein-*S*-yl)acetaminophen protein adducts. The variation in particular fractionation procedures employed by the two studies could account for the difference in adducts quantitated in the nuclear and cell debris fractions. The amount of 3-(cystein-*S*-yl)acetaminophen protein adducts detected immunochemically in the 4000 *g* pellet would be consistent with the concept that components of this fraction are not released into the serum after cell lysis. This is also consistent with the observation that the centrilobular region of mice has been reported to be devoid of cytoplasmic material 3 hr after an hepatotoxic dose of acetaminophen [23]. These findings, as well as our current data, are consistent with the release of 3-(cystein-*S*-yl)acetaminophen protein adducts into the serum from lysed hepatocytes, and the structural components of the hepatocyte cytoskeleton remain localized as persistent adducts.

The requirement for pre-hydrolysis of the mitochondrial, microsomal, and nuclear fractions for efficient antibody recognition indicates that 3-(cystein-*S*-yl)acetaminophen protein adducts in these fractions are not completely accessible to the antibody. In these fractions, the 3-(cystein-*S*-yl)acetaminophen protein adducts are apparently sequestered. This suggests that the NAPQI diffuses into the membrane and covalently binds to integral membrane proteins.

The subcomponents of a 960 *g* pellet were fractionated into mitochondria, nuclei, and plasma membranes and assayed for 3-(cystein-*S*-yl)acetaminophen protein adducts (Fig. 2). The majority of 3-(cystein-*S*-yl)acetaminophen protein adducts in the purified fractions was localized within the mitochondrial and plasma membranes. The early covalent binding in these fractions may have significance to the development of the hepatotoxicity of acetaminophen. The data are consistent with the hypothesis that disruption of Ca^{2+} homeostasis is a critical target for xenobiotic-induced hepatotoxicity [24]. Acetaminophen or NAPQI treatment of isolated hepatocytes has been shown to be associated with thiol depletion and an increase in cytosolic Ca^{2+} [25]. The peak levels of 10 nmol/mg protein of 3-(cystein-*S*-yl)acetaminophen protein adducts in plasma membranes obtained in this experiment are consistent with the findings of Tsokos-Kuhn *et al.* [26], demonstrating substantial radiolabeled covalent binding of acetaminophen metabolities in isolated plasma membranes. Their findings suggest that the decrease in (Ca^{2+},Mg^{2+})-ATPase activity and the arylation of plasma membrane proteins are related to the increase in cytosolic Ca^{2+}. In the future, the identification of the particular acetaminophen protein adducts in the plasma membranes and the relationship of binding to the decrease in Ca^{2+} pump activity should be investigated.

As a result of the recent development of sensitive immunochemical techniques [10, 12, 27, 28], it is now possible to quantitate and investigate the covalent binding of reactive metabolites to proteins. These new methodologies may allow a better understanding of the toxicological insult to cellular macromolecules without the use of radiolabeled compounds. Bartolone *et al.* [27, 29] reported the selective binding of acetaminophen to two polypeptides in mouse liver proteins using Western blots of electrophoretically separated proteins and an antisera with major specificity for the *N*-acetyl group of acetaminophen. The identification of acetaminophen-modified polypeptides and their subcellular localization will require further study. The data presented in this manuscript indicate that the cytosolic fraction contributes to the release of 3-(cystein-*S*-yl)acetaminophen protein adducts into the serum and that the major portion of protein adducts is located in the cytosolic and 4000 *g* pellet fractions. The localization of large quantities of 3-(cystein-*S*-yl)acetaminophen protein adducts in the mitochondrial and plasma membrane fractions may play a critical role in acetaminophen hepatotoxicity.

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