Alterations in the Rat Serum Proteome during Liver Injury from Acetaminophen Exposure

B. Alex Merrick, Maribel E. Bruno, Jennifer H. Madenspacher, Barbara A. Wetmore, Julie Foley, Rembert Pieper, Ming Zhao, Anthony J. Makusky, Andrew M. McGrath, Jeff X. Zhou, John Taylor, and Kenneth B. Tomer


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ABSTRACT

Changes in the serum proteome were identified during early, fulminant, and recovery phases of liver injury from acetaminophen in the rat. Male F344 rats received a single, noninjury dose or a high, injury-producing dose of acetaminophen for evaluation at 6 to 120 h. Two-dimensional gel electrophoresis of immunodepleted serum separated approximately 800 stained proteins per sample from which differentially expressed proteins were identified by mass spectrometry. Serum albumin aminotransferase/asepartate aminotransferase levels and histopathology revealed the greatest liver damage at 24 and 48 h after high-dose acetaminophen corresponding to the time of greatest serum protein alterations. After 24 h, 68 serum proteins were significantly altered of which 23 proteins were increased by >5-fold and 20 proteins were newly present compared with controls. Only minimal changes in serum proteins were noted at the low dose without any alterations. Of the 54 total protein isoforms identified by mass spectrometry, gene ontology processes for 38 unique serum proteins revealed involvement of acute phase response, coagulation, protein degradation, intermediary metabolism, and various carrier proteins. Elevated serum tumor necrosis factor-α from 24 to 48 h suggested a mild inflammatory response accompanied by increased antioxidant capability demonstrated by increased serum catalase activity. Antibody array and enzyme-linked immunosorbent assay analyses also showed elevation in the chemokine monocyte chemoattractant protein-1 and the metalloprotease inhibitor tissue inhibitor of metalloproteinases-1 during this same period of liver injury. This study demonstrates that serum proteome alterations probably reflect both liver damage and a concerted, complex response of the body for organ repair and recovery during acute hepatic injury.

Hepatocellular injury from acetaminophen exposure is primarily initiated by CYP2E1 bioactivation to form reactive intermediates such as N-acetyl-p-benzoquinone imine that deplete glutathione and then bind to critical cellular macromolecules (Park et al., 2005). Mitochondria are thought to be primary targets in acetaminophen toxicity with particular attention on the mitochondrial permeability transition (Kon et al., 2004). Generation of other reactive oxygen species such as nitric oxide and superoxide anion may be important determinants in hepatocyte death (Hinson et al., 2004). Evidence has also been accumulating for the contribution of nonparenchymal cells such as Kupffer cells, natural killer cells, and neutrophils that secrete cytokines and chemokines during acetaminophen-induced liver injury (Lawson et al., 2000; Gardner et al., 2003; Liu et al., 2004). Investigations into the mechanisms of acetaminophen toxicity have been furthered by gene and protein profiling studies of liver using DNA microarrays and proteomic technologies (Fountoulakis et al., 2000; Reilly et al., 2001; Tonge et al., 2001; Ruepp et al., 2002; Heinloth et al., 2004). Acetaminophen treatment in C57BL/6 hybrid mice altered 332 genes and expressed sequence tags by oligoarray expression profiling, including genes involved in stress-response, cell cycle

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ABBREVIATIONS: TNF-α, tumor necrosis factor-α; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CMC, carboxymethylcellulose; ELISA, enzyme-linked immunosorbent assay; MCP-1, monocyte chemoattractant protein-1; IPG, immobilized pH gradient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; MALDI, matrix-assisted laser desorption ionization; ANOVA, analysis of variance; ARP-3, actin-related protein-3; GSH, glutathione; TIMP-1, tissue inhibitor of metalloproteinases-1; LPS, lipopolysaccharide; HGF, hepatic growth factor; APAP, acetaminophen.
and growth inhibition, inflammation, and cell signaling (Reilly et al., 2001). Shared transcript profiles of ATP-dependent genes at subtoxic and toxic doses of acetaminophen in rats indicated the sensitivity of DNA microarrays for identifying adverse effects in the absence of overt toxicity by chemical and histopathology (Heinloth et al., 2004).

Initial proteomic studies on acetaminophen-induced injury have focused upon changes in liver protein expression in mice, identifying known targets for protein adduct formation and also changes in mitochondrial proteins, heat shock proteins, and other structural and intermediary metabolism proteins (Fountoulakis et al., 2000; Tonge et al., 2001; Ruepp et al., 2002). A kinetic approach to acetaminophen toxicity in CD-1 mice from 0.25 to 4 h detected gene transcript changes by DNA microarray or quantitative reverse transcriptase-polymerase chain reaction analyses as early as 15 min postinjection (i.e., granulocyte macrophage-colony-stimulating factor, early growth response-1, and TNF-α) and also changes in mitochondrial proteins, heat shock proteins, and other structural and intermediary metabolism proteins (Welch et al., 2005). After 6 h of 300 mg/kg acetaminophen treatment, 1632 proteins were identified of which SJL mice expressed from 3- to 10-fold higher levels of small ubiquitin-like modifier-1, activating enzyme E1B, complement c5, cyclooxygenase-1, peroxiredoxin 1, Grp170, Hsp70 GSTmu-2, and regucalcin, and other up-regulated proteins with reparative roles. Loss of several mitochondrial proteins from susceptible C57BL/6 mice suggested this organelle was particularly vulnerable to acetaminophen.

Alterations in liver biochemistry and expression profiling are linked in biological context to histopathology and blood chemistry. In particular, blood is one of the most accessible and informative biofluids, not only for specific organ pathology but also for host response to xenobiotic exposure. A comprehensive mapping of soluble human blood elements (i.e., serum or plasma proteome) is currently underway for improved understanding of disease and toxicity (Ommen et al., 2005). Survey of soluble human blood proteins by chromatographic and electrophoretic separation has revealed several thousand resolvable proteins for which mass spectrometry has provided evidence for more than 1000 unique protein identifications (Pieper et al., 2003; Ommen et al., 2005). However, the application of such blood protein maps in pharmacological or toxicity contexts such as serum profiling of liver injury has been limited.

The purpose of the current study was to measure and identify changes in the serum proteomic profile during early, full, and recovery stages of liver injury caused by acetaminophen. Identifying changes in global protein expression of serum proteins will strengthen our understanding of acetaminophen toxicity and recovery in animal models with possible relevance for human exposure.

Materials and Methods

Chemicals. Acetaminophen (99% purity) and sodium carboxymethylcellulose (CMC) were obtained from Sigma-Aldrich (St. Louis, MO). Acetaminophen suspensions were prepared in 0.25% CMC aqueous solution. Monoclonal anti-actin (MAB1501R) was purchased from Chemicon International (Temecula, CA). A polyclonal antibody to aminopeptidase A (sc-18065) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sigma-Aldrich was the source of monoclonal antibody anti-catalase (C0979). ELISA kits to anti-rat TNF-α and anti-rat MCP-1 were provided by BioSource International (Camarillo, CA). A catalase activity assay kit was purchased from Cayman Chemical (Ann Arbor, MI). Rat antibody arrays (catalog no. R0608001) were purchased from RayBiо (Nicross, GA). Bovine serum albumin, β-glycero phosphate, Igepal CA630 (Nonidet P-40), angiotensin II, and adrenocorticotropic hormone fragment 18-39 were purchased from Sigma-Aldrich. The protease inhibitor cocktail Complete, EDTA-free was from Roche Diagnostics (Indianapolis, IN), and the Coomassie Blue Plus protein quantitation reagent was from Pierce Chemical (Rockford, IL). IPG Immobilon dry strips (nonlinear; pH 3-10; 24 cm) were obtained from GE Healthcare, Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Sequencing grade porcine trypsin was purchased from Promega (Madison, WI). The dye SYPRO Orange was acquired from Invitrogen, Molecular Probes (Carlsbad, CA). All other chemicals used were of the highest purity commercially available.

Animals and Animal Care. Male F344/N rats between 10 and 12 weeks of age (250–275 g) were obtained from Taconic Laboratories, Inc. (Raleigh, NC). Three rats were housed in a polycarbonate cage with polyester cage filters (Snow Filtration Co., Cincinnati, OH). Room temperature was maintained at 71–75°F, and humidity was between 36 and 48%. Rats were fed ad libitum with irradiated NTP2000 wafer-feed (Ziegler Brothers, Gardners, PA) and water. Animals were maintained on a 12-h light/dark cycle from 6:00 AM to 6:00 PM.

Experimental Design. Male Fisher F344 rats were fasted for a period of 14 to 16 h before dosing. Water was always available. Rats were dosed between 8:00 AM and 9:00 AM with a volume of 10 ml/kg acetaminophen by oral gavage in an aqueous suspension of 0.25% CMC. Rat chow was returned to cages immediately after dosing.

The selections of doses and exposure times for acetaminophen aimed to characterize the serum proteome at early, fulminant, and recovery stages of liver injury based upon preliminary data and previously published work (Heinloth et al., 2004). Our pilot experiments indicated that 1500 mg/kg reproducibly resulted in centrilobular liver necrosis at 24 h and that one-tenth of this amount at 150 mg/kg represented a subinjury dose. Few changes occurred in serum chemistries or histopathology <12 h (i.e., vacuoles) at these doses. Therefore, in the first proteomic serum study, rats were treated with a single dose of 0, 150, and 1500 mg/kg acetaminophen for 6, 24, or 48 h (n = 5 rats/group) to determine serum proteome changes using two-dimensional gel electrophoresis and mass spectrometry. In a second study to validate and extend initial proteomic findings, rats were treated with 0 and 1500 mg/kg acetaminophen for 24, 72, and 120 h at four rats per group.

Animals were euthanized by carbon dioxide inhalation, and 3 to 5 ml of whole blood was drawn from the inferior vena cava. The liver and right kidney were removed, washed in buffer, and gently blotted. Liver sections were taken from the left and median lobes, and sagittal sections of kidney were made for histopathology. Blood was allowed to clot at room temperature to form serum for 45 min, followed by centrifugation at 3000g for 15 min at 4°C. It is noteworthy that no hemolysis was visible in any samples from control and acetaminophen-treated animals. Serum was removed, sub aliquoted, and stored at −80°C. Aliquots of serum were saved for immunodepletion and two-dimensional gel analysis as well as clinical chemistries. Experiments were performed according to the guidelines established in the National Institutes of Health Guide for the Care and Use of Laboratory Animals by an approved animal study protocol.

Clinical Chemistry and Histopathology. Serum ALT and AST activities were measured by kits from Sigma-Aldrich to assess liver injury in the 6-, 24-, and 48-h study. Since a greater volume of serum was available in the 24-, 72-, and 120-h study, a more...
prehensive serum chemistry analysis could be performed using the Roche Cobas Fara chemistry analyzer (Roche Diagnostics Systems, Inc., Montclair, NJ) that included ALT, AST, total bilirubin, direct bilirubin, total bile acids, alkaline phosphatase, blood urea nitrogen, and creatinine.

After tissues were collected for differential gene expression, cross sections of the left and median lobes were fixed for 24 h in 10% neutral-buffered formalin. Tissues were paraffin-embedded, and hematoxylin and eosin staining was performed. Pathology was scored from 0 to 4 in order of increasing damage: 0, no observed necrosis; 1, a few degenerating parenchymal cells; 2, minimal necrosis; 3, extensive necrosis in which central veins were surrounded by several layers of dead or degenerating cells; and 4, massive necrosis of extensive liver areas.

**Immunodepletion of Serum.** Serum samples were processed by liquid chromatography to remove high-abundance proteins through a protein A/G-based antibody-coupled column specific for rat albumin, transferrin, and IgG in the same manner as described previously for human serum (Pieper et al., 2003). In brief, antibodies to anti-rat albumin (Abcam, Cambridge, MA) and transferrin (Rockland, Gilbertsville, PA) were coupled to POROS A20 and G20 resins (Applied Biosystems, Foster City, CA), respectively. Since the ratio of albumin to transferrin in rat serum is approximately 9:1, the antibody-coupled resins were combined proportionally to this ratio. Eluted serum proteins were neutralized and filtered through Ultrafree-4 centrifugal filter units (Millipore Corporation, Billerica, MA) with a molecular mass cut-off of 5 kDa. After desalting, serum protein concentrates were lyophilized and stored at −80°C until use.

**Western Blot.** After serum immunodepletion, the protein level in each of the serum samples was determined by BCA protein assay (Pierce Chemical). Western blot was performed by SDS-PAGE separation of proteins and electrotransferred by the tank method. In brief, serum samples were diluted at a 1:2 ratio with 4× denaturing sample buffer, boiled for 5 min, and 25 to 50 μg of the serum proteins was loaded onto an 8 to 16% gradient acrylamide gel. After proteins were separated by molecular mass via SDS-PAGE, proteins were transferred onto nitrocellulose by the tank method using Towblyn’s solution. Immunodetection of horseradish peroxidase-labeled secondary antibodies was performed with enhanced chemiluminescence reagents.

**Two-Dimensional Gel Electrophoresis and Image Analysis.** Serum proteins (150 mg) were solubilized in an isoelectric focusing buffer containing 9 M urea, 2% CHAPS, 62.5 mM dithiothreitol, and 2% pH 8 to 10.5 carrier ampholytes to a concentration of 10 μg/μl. Two-dimensional PAGE was performed by the ProGEx system (Large Scale Biology Corporation, Germantown, MD) as described previously, except that proteins were charge separated using IPG strips (Pieper et al., 2003). In brief, 150-μg samples were separated on IPG 24-cm strips (GE Healthcare) at pH 3 to 10 on an IPGphor unit (GE Healthcare). A step-and-hold voltage protocol was used overnight for a total of 68,728-V h. When isoelectric focusing was complete, the strips were reeled for mass separation by reduction and alkylation (6 M urea, 2% SDS, 0.375 M Tris, pH 7.5, 30% glycerol, and 0.078 M dithiothreitol or 0.065 M iodoacetamide) by gentle rocking for 30 min in each solution. For separation by mass, slab gels at 1-mm thickness were cast with Angelique, a computer-controlled gradient casting system forming an 11 to 19% T acrylamide gradient. IPG strips were gently slid onto the top of slab gels and run in DALT tanks in SDS-PAGE buffer (0.00374 M SDS, 0.0240 M Tris-HCl, and 0.2 M glycine) at 10°C until the tracking dye front reached the bottom of the gel (approximately 4300 V-h). Gels were stained with SYPRO Orange (Invitrogen) using an automated staining and scanning system. Stained gels were scanned as TIFF images that were analyzed to generate a spot list, giving position, shape, and density information to determine differential protein expression using Kepler software (Large Scale Biology Corporation). Mean values for each protein were used to calculate -fold change from control.

**Mass Spectrometry Identification of Proteins.** The procedures for protein retrieval from gel plugs and digestion with trypsin were carried out by applying standard procedures with robotic devices. MALDI targets were automatically run on a Bruker Biflex or Autoflex mass spectrometer (Bruker, Bremen, Germany). Both instrument models were equipped with delayed ion extraction, pulsed nitrogen lasers, dual microchannel plates, and 2-GHz transient digitizers. All mass spectra represented signal averaging of 120 laser shots. The performance of the mass spectrometers produced sufficient mass resolution to produce isotopic multiplets for each ion species below m/z 3000. Spectra were internally calibrated using two spiked peptides (angiotensin II and adrenocorticotropic hormone fragment 18-39) and database-searched with a mass tolerance of 50 or 100 ppm. LC-MS/MS analysis was conducted using a Finnigan LCQ mass spectrometer (Thermo Electron Corporation, Waltham, MA), equipped with a microelectrospray interface. Spectra were acquired in automated tandem mass spectrometry mode in which additional parameters of dynamic exclusion, isotopic exclusion and top three ions were incorporated into the procedure. The scan range for mass spectrometry mode was set at m/z 375 to 1400. A parent ion default charge state of +2 was used to calculate the scan range for acquiring tandem mass spectrometry data.

Mass spectrometry data were automatically registered, analyzed, and searched against the SwissProt and National Center for Biotechnology Information public protein databases using RADARS, a separate relational database (Harvard Apparatus Inc., Holliston, MA), and optimized in-house at Large Scale Biology Corporation. For MALDI peptide mapping, MASCOT (Matrix Science Ltd., London, UK) and ProFound (Harvard Apparatus Inc.) search engines were used. Identifications for MALDI data were registered when search results were above the 95th percentile of significance in both ProFound and MASCOT. MASCOT was used for peptide sequence searching of LC-MS/MS data in which scores above the 95th percentiles (MASCOT ≥50) qualified for protein identification.

**Statistical Analysis.** The Wilcoxon rank sum test was used for two-dimensional gels to detect pairwise quantitative changes in protein abundance (integrated fluorescent intensity) between the treatment groups and their respective time-matched control group at a significance level of p ≤ 0.01. It was also required that the magnitude of -fold change be ≥ 1.5 times. All changes between groups were visually verified for correct matching with the master gel image. Biochemical data were analyzed by ANOVA using Duncan’s post hoc test for comparisons among means at p ≤ 0.05 when appropriate.

**Results**

**Acetaminophen Liver Injury at 6, 24, and 48 h.** Serum protein profiling of acetaminophen-induced liver injury was performed at doses and times of exposure designed to produce histopathology reflecting early, fulminant, and subsiding stages of hepatic damage at 6, 24, and 48 h. A low dose at 150 mg/kg acetaminophen was tested at 0.1-fold of the liver injury-producing dose (1500 mg/kg) to determine whether changes could be found by image analysis of serum proteins separated by two-dimensional gel electrophoresis for each animal. As expected, no detectable histopathological changes were observed at 150 mg/kg acetaminophen nor were clinical chemistries altered from control (data not shown). However, notable histopathological and serum protein changes were observed at the higher dose of 1500 mg/kg acetaminophen from 6 to 48 h. Results in Fig. 1 show representative histopathology at 6, 24, and 48 h at the 1500-mg/kg acetaminophen dose. The associated numbers of serum protein changes at each time point are also presented in Fig. 1 for the low (150 mg/kg) and high dose (1500 mg/kg) of acetaminophen. After 8 h of 1500 mg/kg acetaminophen, hepatocytes in centrilo-
lar areas showed some degenerative changes manifested by cytoplasmic microvacuoles in centrilobular hepatocytes in four of five total rats. At 24 h, acetaminophen treatment produced marked centrilobular necrosis combined with sinusoidal erythrocytic congestion in all animals, which declined by 48 h at which time only three of five rats showed residual centrilobular congestive necrosis. Serum clinical chemistries corroborated the histopathology and were expressed as mean -fold increases in ALT/AST ($n=5$/group). The ALT and AST values of vehicle controls were 95 and 125 U/l at 6 h, 91 and 126 U/l at 24 h, and 74 and 102 U/l at 48 h. At 1500 mg/kg acetaminophen, the ALT/AST values showed no change at 6 h, were 6- and 86-fold increased above time-matched controls at 24 h, and were 25- and 29-fold increased above time-matched controls at 48 h. Histopathology and ALT and AST clinical chemistries suggested 1500 mg/kg acetaminophen produced phenotypes of early, full, and recovery stages of liver injury at 6, 24, and 48 h, respectively, and that 150 mg/kg was a subinjury dose.

**Proteomic Analysis of Serum.** Proteomic analysis of serum by two-dimensional gel separation corresponded with the severity of liver injury. The numbers of proteins resolved by two-dimensional gels that were altered from control by acetaminophen treatment are shown for 6, 24, and 48 h (Fig. 1, bar graph). A dose of 150 mg/kg acetaminophen produced a discrete number of serum protein changes from control (two proteins at 6 h, three proteins at 24 h, and no proteins at 48 h). Although only one protein was altered at 6 h, administration of 1500 mg/kg, acetaminophen significantly altered 68 serum proteins after 24 h of which 23 proteins were >5-fold change and 20 proteins were newly detectable (i.e., not in controls) in serum after acetaminophen treatment. By 48 h, the number of altered serum proteins declined to 17 proteins of which only one protein was >5-fold changed and only two proteins were newly found with treatment versus control. Therefore, two-dimensional gel separation and image analysis detected minimal changes at 6 h, the greatest number and magnitude of serum protein alterations at 24 h, with a decline in serum protein changes at 48 h after a liver injury dose of 1500 mg/kg acetaminophen.

Serum proteins that were significantly altered ($p<0.01$) by acetaminophen treatment were excised from two-dimensional gels, digested, and identified by mass spectrometry as shown in Table 1. Serum proteins were electronically registered to common coordinates and were represented by master spot numbers in the common coordinate (master) gel. Table 1 contains the -fold change versus time-matched control of identified proteins (UniProt accession number) after various times for either the 150 or 1500 mg/kg acetaminophen dose. For the 68 protein isoforms changed at 24 h, 41 (60%) were identified by mass spectrometry. At 48 h, 14 proteins (82%) of 17 altered proteins were identified. Figure 2A displays an electronic two-dimensional gel image of total separated serum proteins at 24 h that have been annotated with identified proteins according to Table 1. Several proteins were detected as separate isoforms that were reproducibly resolved in differential expression analysis after acetaminophen treatment, including fetuin B, Gc globulin, complement C3, antithrombin-III, hemopexin, actin, apolipoprotein E, and others. Some proteins were not detectable in control but
TABLE 1
Altered serum proteins with acetaminophen treatment in rats
Differentially expressed serum proteins after APAP exposure in male rats found after proteomic analysis. Rats were exposed to 0 (vehicle), 150, and 1500 mg/kg APAP for 6, 24, or 48 h at n = 5/group. Sera were collected for two-dimensional gel separation and mass spectrometry analysis. Serum from each rat was immunodepleted of albumin, Igs, and transferrin before two-dimensional gel electrophoresis of 150 μg of serum protein. Image analysis determined differential protein expression among time-matched control and treated animals before protein identification by mass spectrometry according to the common name as referenced by the UniProt accession number (http://www.pi.r/pi.r). Scanned images of all two-dimensional gels were registered to a common x,y coordinate system to which proteins were assigned MSNs. In some cases, multiple protein isoforms were analyzed as the same protein identity by mass spectrometry, which is indicated by different MSNs for the same accession number (http://www.pir.uniprot.org/). For newly appearing proteins in serum from acetaminophen-treated rats that were not detected in control rats (ND Ctl), no -fold change was assigned.

<table>
<thead>
<tr>
<th>MSN</th>
<th>Treatment/Time</th>
<th>Accession No.</th>
<th>GO Ontology No.; Process/Function; Location</th>
<th>-Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>145</td>
<td>Fetuin-B</td>
<td>Q9QX79</td>
<td>Cys protease inhibitor, HGF, insulin regulation, inflam response; EC space</td>
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</tr>
<tr>
<td>176</td>
<td></td>
<td></td>
<td></td>
<td>1.8</td>
</tr>
<tr>
<td>223</td>
<td></td>
<td></td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>431</td>
<td>Ge-globulin (vitamin D-binding protein)</td>
<td>P04276</td>
<td>Transport/actin, VitD binding; EC space</td>
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</tr>
<tr>
<td>558</td>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>228</td>
<td>Haptoglobin</td>
<td>P06866</td>
<td>Proteolysis/Hb binding; EC space</td>
<td>2.4</td>
</tr>
<tr>
<td>293</td>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
<tr>
<td>365</td>
<td>Complement C3 (C3)</td>
<td>P01026</td>
<td>Cys protease inhibitor, HGF, insulin regulation, inflam response/endopeptidase inhibitor; EC space</td>
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<tr>
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<td>Transthyretin</td>
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<td>heparin binding; EC space</td>
<td>1.9</td>
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<tr>
<td>337</td>
<td>Actin, cytoplasm</td>
<td>P48975</td>
<td>cytoskeleton, biogenesis/cellular structure; cytoskeleton, cytoplasm</td>
<td>1.8</td>
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<tr>
<td>606</td>
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<td></td>
<td></td>
<td>2.1</td>
</tr>
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<td>399</td>
<td>Heat shock protein 70</td>
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<td>protein folding; cellular activation, inflam response/endopeptidase inhibitor; EC space</td>
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<td>P04916</td>
<td>protein metabolism; cytosol</td>
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<tr>
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<td>Complement C4A (anaphylatoxin)</td>
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<td>complement activity, inflam response/chemotaxis; EC space</td>
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<td>Urocanase (urocanate hydratase)</td>
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<td>histidine catabolism/lyase; cytoplasm</td>
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<td>ALT</td>
<td>P25409</td>
<td>biosynthesis/Ala transaminase; cytoplasm</td>
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<tr>
<td>705</td>
<td>Glycine N-methyl-transferase (GlyNMeTx)</td>
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<td>SAM, FA metabolism/methyl transferase; cytoplasm</td>
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<td>α-1-Inhibitor III (α-1-Inh-III)</td>
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<td>acute phase response/protease inhibitor; EC space</td>
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<td>nucleic acid metabolism/deaminase; cytoplasm</td>
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<td>P06214</td>
<td>heme synthesis/metal binding; primarily cytoplasm</td>
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<tr>
<td>800</td>
<td>Catalase</td>
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<td>Acyl-CoA dehydrogenase long-chain specific, mitochondrial precursor</td>
<td>P15650</td>
<td>lipid metabolism/acyl-CoA deHase; mitochondria</td>
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were newly found in serum after acetaminophen treatment such as argininosuccinate synthase, urocanase, glycine N-methyltransferase, acyl-CoA dehydrogenase, actin-related protein-3 (ARP-3), GSH synthetase, and others. Although a modest -fold change (<5×) occurred in many proteins, acetaminophen effects were more pronounced (>5×) with an increase or decline of proteins in serum, such as β-actin, glutamate dehydrogenase, alamine aminotransferase, aldehye dehydrogenase, guanine deaminase, catalase, and ornithine decarboxylase. It is notable that multiple isoforms of 11 serum proteins, such as complement C3, were found to be altered during liver injury.

Gene ontology terms (Gene Ontology Consortium; http://www.ebi.ac.uk/GOA/) of identified serum proteins in Table 1 represent cellular enzymes in protein degradation, intermediary metabolism, and carrier proteins that occur at higher serum levels during liver injury as well as the appearance of inflammatory, acute phase, and coagulatory proteins. Bar graphs in Fig. 2B show relative intensity levels of selected proteins identified from two-dimensional gels for each individual animal in the group (n = 5). For some proteins, there was a basal level of detectable amounts in controls (i.e., glutamate dehydrogenase, aminopeptidase, catalase and carbonic anhydrase II), whereas other proteins were only detectable after liver injury such as ARP-3, GSH synthetase, adenosylhomocysteinase, and 4-hydroxyphenylpyruvate dioxygenase.

**Western Blot of Serum Proteins Altered by Acetaminophen.** Selected serum proteins altered by 1500 mg/kg acetaminophen treatment at 24 h were validated by Western blot with available antibodies (Fig. 3). Acetaminophen-treated rats exhibited widespread necrosis, and ALT (>4000 U/l) values were increased above control for acetaminophen rats (lanes 6 and 8–10), although the ALT value for the rat in lane 7 was not as high (947 U/l). Immunodepleted sera for individual rats in each group were loaded into each lane for separation and immunodetection. Two isoforms of aminopeptidase were found by Western blotting from which the 90-kDa form (top blot) was most responsive to acetaminophen for three of five rats (lanes 8–10). The 160-kDa form was not substantially affected by treatment (data not shown). Appearance of serum catalase and β-actin were apparent in four of five rats after 1500 mg/kg acetaminophen treatment (lanes 6 and 8–10). With the exception of aminopeptidase, signals in control lanes 1 to 5 were minimal for all serum proteins examined.

We wanted to test the hypothesis that catalase as detected by Western blot was catalytically active in serum during the period of acetaminophen injury. However, data from the 6- to 48-h experiment suggested that a 48-h treatment period might not be long enough to evaluate the effects of acetaminophen. In addition, biological process and functional categories of identified serum proteins in Table 1 suggested the presence of underlying inflammatory processes associated with liver injury after the high dose of acetaminophen. These reasons provided a rationale for conducting a second acetaminophen exposure experiment using only 1500 mg/kg acetaminophen to study effects of liver injury and organ recovery for up to 5 days. Since the requirements of sufficient serum for proteomic separations in the initial study precluded some biochemical analyses, the second study was able to include a wider panel of serum chemistries and follow-up analyses as well as histological evaluation.

**Acetaminophen Liver Injury from 24 to 120 h.** In a second study, rats exposed to 1500 mg/kg acetaminophen showed signs of acute liver injury (Fig. 4) and ALT/AST elevations after 24 h as before. Evaluation of histopathology after acetaminophen at 24 h showed centrilobular necrosis (mean score of 2.5 in four of four rats) with sinusoidal erythrocytic congestion that was diminished after 72 h (mean necrosis score of 1.75 in four of four rats), which had resolved.

<table>
<thead>
<tr>
<th>MSN</th>
<th>Protein Identification</th>
<th>Accession No.</th>
<th>GO Ontology^a</th>
<th>^ Function (with GO terms)</th>
<th>Location</th>
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<tr>
<td>965</td>
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<td>P32755</td>
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<td>1483</td>
<td>Adenosylhomocysteinase</td>
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<td>metabolism/dioxygenase; cytoplasm</td>
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<tr>
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to no necrosis in any rats by 120 h (data not shown). In addition, discrete areas of mineralization were also observed in liver sections at 72 and 120 h after acetaminophen (data not shown). Figure 4 shows that the increased levels of ALT and AST levels observed after 24 h of acetaminophen had greatly declined by 72 h, but peaks of serum bilirubin, bile acids, and alkaline phosphatase occurred at this time. These serum changes suggested impairment of liver function at 72 h after acetaminophen despite a decline in ALT/AST. However, by 120 h, serum clinical values had nearly returned to normal or slightly below normal for all serum indicators of liver function (Fig. 4). It is noteworthy that no premature animal deaths occurred before sacrifice in either time-course study.

Increased Serum Catalase Levels and Activity in Acetaminophen Liver Injury. Serum catalase was measured by Western dot blot to confirm two-dimensional gel results and permit comparisons of individual rat serum samples from both experiments as shown in Fig. 5. A single band for catalase was observed by Western blot, so dot blot signal could be attributed to this protein. Minimal catalase differences were detected by Western blot at 6 h, but four of five rats were positive for catalase by 24 h and all five rats showed the presence of catalase at 48 h after 1500 mg/kg acetaminophen. In the second experiment, serum catalase was found in all rats after 24 h (three prominently), but signal was reduced at 72 h and was similar to controls by 120 h. In the adjacent panel of Fig. 5, mean serum catalase activities were consistent with Western dot blotting results and were highest at 24 and 48 h and declined by 72 and 120 h.

Antibody Array and Cytokine Analysis in Acetaminophen Liver Injury. Proteomic analysis of serum suggested the involvement of inflammatory processes during liver injury from acetaminophen. We further tested this possibility using ELISA assays and cytokine antibody arrays. Since TNF-α is a frequent mediator of inflammation, it was measured by quantitative ELISA assay at 6 to 48 h and 24 to 120 h after acetaminophen. TNF-α levels increased as early as 6 h in the first experiment (Fig. 6, left) and continued to rise at 24 and 48 h after acetaminophen. In the second experiment, serum catalase was found in all rats after 24 h (three prominently), but signal was reduced at 72 h and was similar to controls by 120 h. In the adjacent panel of Fig. 5, mean serum catalase activities were consistent with Western dot blotting results and were highest at 24 and 48 h and declined by 72 and 120 h.

Antibody arrays provided a means to screen for altered
serum levels of cytokines and chemokines in inflammatory reactions. Pooled sera from animals of each group were incubated with membrane-immobilized, rat-specific antibodies overnight at 4°C. Captured cytokines and chemokine ligands were detected by chemiluminescence. Membranes for each control and acetaminophen treatment groups for 6 to 48 h were incubated along with a blank membrane (no rat sera). Chemiluminescent detection was simultaneously performed with all membranes (seven membranes/experiment) for intercomparability among treatment groups in each experiment of 6 to 48 or 24 to 120 h. Two serum factors, MCP-1 and TIMP-1, were found to be consistently increased above controls during acetaminophen treatment (Fig. 7, A and B).

The findings from antibody arrays were confirmed for MCP-1 (Fig. 7C) by quantitative ELISA using serum from each animal. ELISA results showed no change from control at 6 h, but substantial increases in serum MCP-1 concentrations were detected at 24 and 48 h after acetaminophen treatment. In the second experiment in which rats were
exposed to 1500 mg/kg acetaminophen at 24, 72, and 120 h, the increases in MCP-1 concentration at 24 h were pronounced but returned to just above control levels at 72 and 120 h. These results suggest that the highest levels for serum MCP-1 were observed at 24 to 48 h after acetaminophen treatment.

**Discussion**

In this study, two-dimensional gel electrophoresis and mass spectrometry were used to profile serum proteins during acute liver injury and recovery in rats after acetaminophen exposure. More than 800 densiometric features were reproducibly detected on each two-dimensional gel for each animal. Serum proteins as measured by differential two-dimensional gel electrophoresis seemed remarkably stable in the absence of liver injury since so few changes were observed from 6 to 48 h at the lower dose of acetaminophen or at 6 h after an injury-producing dose. However, during the time of peak toxicity with a known liver injury dose of acetaminophen as determined by histopathology and ALT/AST activities, differential proteomic analysis showed the greatest number of serum protein changes. Of the 68 gel features that were altered, almost 80% were identified by mass spectrometry. Many of these altered serum proteins in acetaminophen-induced liver injury have functions in acute phase response, coagulation, scavenging, transport, intermediary metabolism, catabolism, or structural functions. By 48 h, the number of serum protein changes were reduced by 4-fold compared with those observed at 24 h, clinical serumchemistries had declined, and histological features in the hepatic centrilobular region indicated regenerative processes were well underway.

There have been only limited proteomic studies of acetaminophen liver injury in rats despite the large body of literature in this species (Park et al., 2005). Liver and serum were probed by two-dimensional electrophoresis in Sprague-Dawley rats for new markers of hepatomegaly, hepatic necrosis, or hepatobiliary injury after single exposures to four compounds, including phenobarbital and Wyeth-14,643 (pirinixic acid), acetaminophen, and /H9251-naphthylisothiocyanate, respectively (Amacher et al., 2005). Similar to the current study, they noted that 1000 mg/kg acetaminophen increased serum 4-hydroxyphenylpyruvate dioxygenase, and of 19 altered serum proteins, these four agents increased Gc globulin and malic dehydrogenase and also decreased plasma retinal-binding protein. A related proteomic study similarly examining various hepatotoxicants identified 100 to 200 rat liver proteins, including reduced levels of hepatic catalase as a common finding (Thome-Kromer et al., 2003), although this observation remains unexplained.

A primary interest in serum proteomic analysis during acetaminophen hepatotoxicity is to increase understanding of drug-induced liver injury by detection of serum protein changes. A progression of critical events starting from reactive intermediate formation, protein adducts, alterations in hepatocellular gene expression, biochemistry, and function eventually affects the serum proteome. Genomic analysis has shown transcript changes are detectable at early times (15
indirect consequence of liver necrosis. Vascular endothelia from oxidative injury that occurs as an
phen toxicity as a beneficial host response for protecting
(Leff et al., 1991). Importantly, therapeutics with catalase
jects that contain increasing amounts of catalase activity
from hydrogen peroxide by the serum of normal human sub-
studies report that cultured endothelia are better protected
in vitro experiments with hydrogen peroxide. Previous
(Laskin, 1996), lung (Hammerschmidt and Wahn, 2004)
oxidative vascular damage upon endothelia that line the liver
present in serum at 72 h during recovery. The potential for
ing to greatest liver injury. However, catalase was still
biochemistry at 24, 48, and 72 h demonstrated that it was
enzymatically most active at 24 to 48 h at times correspond-
ing to greatest liver injury. However, catalase was still
present in serum at 72 h during recovery. The potential for
oxidative vascular damage upon endothelia that line the liver
(Laskin, 1996), lung (Hammerschmidt and Wahn, 2004)
brain (Li et al., 2003), and other organs has been documented
by in vitro experiments with hydrogen peroxide. Previous
studies report that cultured endothelia are better protected
from hydrogen peroxide by the serum of normal human sub-
jects that contain increasing amounts of catalase activity
(Leff et al., 1991). Importantly, therapeutics with catalase
activity have been shown to prevent sinusoidal and hepato-
cellular damage in models of liver injury (Yabe et al., 1999).
We interpret increased serum catalase during acetaminophen
toxicity as a beneficial host response for protecting
vascular endothelia from oxidative injury that occurs as an
indirect consequence of liver necrosis.

The actions of cytokines, inflammatory mediators, and re-
active oxidant species have gained increasing prominence for
their roles in acetaminophen-induced liver injury (Dambach
et al., 2005; James et al., 2005b). Acute phase response pro-
teins that we found in serum suggested we search for other
serum cytokines and chemokines, particularly since these
are typically below detection limits for staining in two-di-
ensional gels. We complemented our proteomics strategy
with cytokine antibody arrays and ELISAs to survey further
serum protein changes. Serum TNF-α levels peaked at 24 to
48 h after acetaminophen but remained increased above control
up to 120 h. Likewise, antibody array and ELISA data in
our study showed a 24- to 48-h peak for MCP-1 and TIMP-1
after acetaminophen in a manner that might reflect a coordi-
ation of reparative processes. Proteolytic degradation of
the extracellular matrix is essential for tissue remodeling in
liver repair, involving a combination of matrix metallopro-
teinases and their specific tissue inhibitors or TIMPs (Rud-
dolph et al., 1999). A major factor in liver regeneration is the
release and activation of pro-HGF from extracellular matrix
via proteolytic cleavage (Liu et al., 1994). TIMP-1 is thought
to play a central role in regulating release and activation of
HGF via the TIMP/metalloproteinase proteolytic axis that
ultimately controls the HGF-mitogenic signaling pathway
(Mohammed et al., 2005). Although the exact roles of inflam-
lation and increased serum proteins such as catalase,
MCP-1, and TIMP-1 as contributory or restorative factors in
acute liver injury are controversial (Gardner et al., 2003; Liu
et al., 2004; Jaeschke et al., 2005), immune involvement
could represent a systemic host defense mechanism to organ
injury.

The current capabilities of proteomic technologies com-
bined with the wide quantitative range and complexity of
serum protein expression often require use of multiple ex-
pression platforms as used in this study. Equally important
are prepurification strategies such as immunodepletion to
remove the abundant, less informative serum proteins and
allow more facile detection of low-concentration serum pro-
teins by proteomic methods. In this rat model, we observed
relatively few changes in our probing of the serum proteome
in the absence of tissue injury at a subtoxic dose of 150 mg/kg
from 6 to 48 h and with a toxic dose at 1500 mg/kg acetamin-
ophen at 6 h. This apparent stability of the serum proteome
is in part due to proteomic platform limits in sensitivity, but
it is also consistent with other rodent studies in which liver
transcript alterations precede changes in clinical chemistries
or cytokines levels at injury-producing acetaminophen doses
(Dambach et al., 2002; James et al., 2005a) or even subtoxic
doses (Heinloth et al., 2004). As proteomic technologies im-
prove and coverage of the rat serum proteome becomes
deeper and better defined, it is probable that more subtle
changes in low-level regulatory proteins will be observed
earlier in the damage process as the animal adjusts to liver
injury. Many of the reported proteome changes we observed
consisted of passively released cellular contents from dam-
aged hepatocytes as well as actively secreted signaling pep-
tides (cytokines and chemokines) from both resident non-
parenchyma (Kupper, stellate, and pit cells) and recruited
circulating leukocytes. It is also possible that other organs
and tissues actively contribute or secrete proteins as new
serum constituents in the face of major organ damage since
blood comes in contact with all organs and tissues of the host.
Complete description of the serum proteome of the rat would
advance an understanding of the biology of host response to
liver injury in an important preclinical species while providing new markers that might distinguish between pharmacological effects and injurious effects of therapeutic agents.

References


Address correspondence to: Dr. B. Alex Merrick, National Institute of Environmental Health Sciences, D2-04, P.O. Box 12233, Research Triangle Park, NC 27709. E-mail: merrick@niehs.nih.gov