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

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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, non-injury dose or a high, injury-producing dose of acetaminophen for evaluation at 6 hr to 120 hr. Two-dimensional gel electrophoresis of immunodepleted serum separated about 800 stained proteins per sample from which differentially expressed proteins were identified by mass spectrometry. Serum ALT/AST levels and histopathology revealed the greatest liver damage at 24 and 48 hr after high dose acetaminophen corresponding to the time of greatest serum protein alterations. After 24 hr, 68 serum proteins were significantly altered of which 23 proteins were increased by >5 fold and 20 proteins were newly present compared to controls. Only minimal changes in serum proteins were noted at the low dose without any histopathology. 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 TNF α from 24 to 48 hr suggested a mild inflammatory response accompanied by increased antioxidant capability demonstrated by increased serum catalase activity. Antibody array and ELISA analyses also showed elevation in the chemokine, MCP-1, and the metalloprotease inhibitor, TIMP-1, during this same period of liver injury. This study demonstrates that serum proteome alterations likely reflect both liver damage and a concerted, complex response of the body for organ repair and recovery during acute hepatic injury.

Abbreviations

ACTH₁₈₋₃₉, adrenocorticotrophic hormone fragment 18-39; ALT, alanine aminotransferase; ANIT, α -naphthylisothiocyanate; ARP3, actin related protein-3; AST, aspartate aminotransferase; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; CINC-2 and -3, Cytokine induced neutrophil chemoattractant-2 and -3; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol-bis(β -aminoethylether)-N,N',N'-tetra-acetic acid; ECL, enhanced chemiluminescence; ECM, extracellular matrix; EGF-1, epidermal growth factor-1;

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ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; GST, glutathione S-transferase; GM-CSF, granulocyte-macrophage colony stimulating factor; GRP, glucose responsive protein; HGF, hepatic growth factor; HEPES, N-2-Hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid; 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase; HRP, horseradish peroxidase; ICAT, isotope coded affinity tags; IPG, immobilized pH gradients; IEF, isoelectric focusing; LC, liquid chromatography; LIX, LPS-induced C-X-C chemokine; MALDI, matrix assisted laser desorption ionization; MCP1, monocyte chemoattractant protein-1; MS/MS, tandem mass spectrometry; Mr, relative molecular mass; MS, mass spectrometry; MSN, master spot number; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SUMO1, small ubiquitin-like modifier-1; TIMP-1, tissue inhibitor of metalloproteinases-1; TNF α , tumor necrosis factor-alpha

Introduction

Hepatocellular injury from acetaminophen exposure is primarily initiated by Cyp2E1 bioactivation to form reactive intermediates such as N-acetyl-p-benzoquinone imine (NAPQI) 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 non-parenchymal 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 C57B1/6 hybrid mice altered 332 genes and ESTs by oligoarray expression profiling including genes involved in stress-response, cell cycle 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 clinical chemistries 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 – 4 hr detected gene transcript changes by DNA microarray or qRT-PCR analyses as early as 15 min post-injection (i.e. GM-CSF, EGR-1, TNF α) and 20 hepatic protein alterations by two-dimensional gels and mass spectrometry during the 4 hr period (Ruepp et al., 2002). A recent study exploited inherent differences in acetaminophen toxicity between resistant SJL and susceptible C57B1/6 mouse strains to gain insight into hepatotoxicity using LC-MS/MS analysis by ICAT of liver proteins (Welch et al., 2005). After 6 hr of 300 mg/kg of acetaminophen treatment, 1632 proteins were identified of which SJL mice expressed from 3–10 fold higher levels of SUMO1, activating enzyme E1B, complement c5, cyclooxygenase-1, peroxiredoxin 1, Grp170, Hsp70 GST μ -2 and regucalcin, and other upregulated proteins with reparative roles. Loss of several mitochondrial proteins from susceptible C57B1/6 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 chemistries. 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 (Omenn et al., 2005). Survey of soluble human blood proteins by chromatographic and electrophoretic separation have revealed several thousand resolvable proteins for which mass spectrometry has provided evidence for over 1000 unique protein identifications (Pieper et al., 2003; Omenn et al., 2005). However, the application of such blood protein maps in pharmacologic 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.

Methods

Chemicals

Acetaminophen (99% purity) and sodium carboxymethylcellulose (CMC) were obtained from Sigma Chemical Company (St. Louis, MO). Acetaminophen suspensions were prepared in 0.25% CMC aqueous solution. Monoclonal anti-actin (MAB1501R) was purchased from Chemicon (Temecula, CA). A polyclonal antibody to aminopeptidase A (sc-18065) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sigma-Aldrich (Saint Louis, MO) was the source of MAb 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 (Ann Arbor, MI). Rat antibody arrays (Cat No. R0608001) were purchased from RayBio (Norcross, GA). Bovine serum albumin, β -glycerylphosphate, Ipegal CA630 (Nonidet P-40), angiotensin II, and ACTH_{18–39} (adrenocorticotrophic hormone fragment 18–39) were purchased from Sigma-Aldrich (St. Louis, MO). The protease inhibitor cocktail CompleteTM, EDTA-free was from Roche Diagnostics (Indianapolis, IN) and the Coomassie Plus protein quantitation reagent was from Pierce Chemicals (Rockford, IL). IPG Immobiline dry stripsTM (non-linear, pH 3–10, 24 cm) were obtained from GE Healthcare, Amersham Biosciences (Piscataway, NJ). Sequencing grade porcine trypsin was purchased from Promega (Madison, WI). The dye SYPROTM Orange was acquired from Invitrogen, Molecular Probes (Eugene, OR). All other chemicals used were of the highest purity commercially available.

Animals and Animal Care

Male F344/N rats between 10 to 12 weeks of age (250–275g) 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° to 75°F, and humidity between 36 to 48%. Rats were fed *ad libitum* with irradiated NTP2000 wafer feed (Ziegler Brothers, Gardners, PA) and water. Animals were maintained on a 12 hr light-dark cycle from 0600–1800 hr.

Experimental Design

Male, Fisher F344 rats were fasted for a period of 14–16 hrs prior to dosing. Water was always available. Rats were dosed between 0800 to 0900 hr with a volume of 10 ml/kg of 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 hr and that one-tenth of this amount at 150 mg/kg represented a sub-injury dose. Few changes occurred in serum chemistries or histopathology <12 hr (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 of acetaminophen for 6 hr, 24hr or 48hr (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 hr at 4 rats per group.

Animals were euthanized by carbon dioxide inhalation and 3–5 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 3000xg for 15 min at 4°C. Notably, no hemolysis was visible in any samples from control and acetaminophen treated animals. Serum was removed, subaliquoted 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 NIH Guide for the Care and Use of Laboratory Animals by an approved animal study protocol.

Clinical Chemistries and Histopathology

Serum ALT and AST activities were measured by kits from Sigma (St. Louis, MO) to assess liver injury in the 6, 24, 48 hr study. Since a greater volume of serum was available in the 24, 72 and 120 hr study, a more comprehensive serum chemistry analysis could be performed using the Roche Cobas Fara chemistry analyzer (Roche Diagnostic 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 hr in 10% neutral buffered formalin. Tissues were paraffin-embedded and hematoxylin-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 immunoglobulin G (IgG) in the same manner as previously described for human serum (Pieper et al., 2003). Briefly, 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 about 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, Billerica, MA) with a molecular weight cutoff of 5kD. 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 the Pierce BCA (bicinchoninic acid) assay (Pierce, Rockford, IL). Western blot was performed by SDS PAGE separation of proteins and electrotransferred by the tank method.. Briefly, serum samples were diluted at a 1:2 ratio with 4X denaturing sample buffer, boiled for 5 minutes and 25–50µg of the serum proteins were loaded onto an 8–16% gradient acrylamide gel. After proteins were separated by molecular weight via SDS-PAGE, proteins were transferred onto nitrocellulose by the tank method using Towbin's solution. Immunodetection of HRP-labeled secondary antibodies was performed with ECL reagents.

Two-dimensional Gel Electrophoresis and Image Analysis

Serum proteins (150 mg) were solubilized in an IEF buffer containing 9M urea, 2% CHAPS, 62.5 mM dithiothreitol and 2% pH 8–10.5 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 previously described except that proteins were charge separated using IPG strips (Pieper et al., 2003). Briefly, 150 µg samples were separated on IPG 24 cm strips (GE Healthcare) at pH 3 to 10 on an IPGphor™ unit (GE Healthcare, Amersham Biosciences, Piscataway, NJ). A step-and-hold voltage protocol was used overnight for a total of 68,728V-hrs. When isoelectric focusing was complete, the strips were readied for mass separation by reduction and alkylation (6M urea, 2% SDS, 0.375M Tris pH 7.5, 30% glycerol, and 0.078M dithiothreitol or 0.065M iodoacetamide) by gentle rocking for 30 minutes in each solution. For separation by mass, slab gels at 1mm thickness were cast with Angelique™, a computer-controlled gradient casting system forming an 11–19%T acrylamide gradient. IPG strips were gently slid onto the top of slab gels and run in DALT tanks in SDS-PAGE buffer (0.00374M SDS, 0.0240M Tris-HCl and 0.2M glycine) at 10°C until the tracking dye front reached the bottom of the gels (approximately 4300Vh). Gels were stained with SYPRO™ Orange (Invitrogen, Molecular Probes, Eugene, OR) 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™ (LSBC, Germantown, MD). 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 (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 ACTH_{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 Finnigan, San Jose CA), equipped with a micro-electrospray interface. Spectra were acquired in automated MS/MS mode in which additional parameters of dynamic exclusion, isotopic exclusion and top-3-ions were incorporated into the procedure. The scan range for MS mode was set at m/z 375–1400. A parent ion default charge state of +2 was used to calculate the scan range for acquiring MS/MS data.

Mass spectrometry data were automatically registered, analyzed and searched against the SwissProt and NCBI public protein databases using RADARS™, a separate relational database (Harvard Biosciences, Holliston, MA), and optimized in-house at LSBC. For MALDI peptide mapping, MASCOT™ (Matrix Science, London, UK) and ProFound™ (Harvard Biosciences,

Hollister, MA) search engines were employed. 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 percentile (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 $p \leq 0.01$. It was also required that the magnitude of fold change be $\geq 1.5X$. All changes between groups were visually verified for correct matching with the master gel image. Biochemical data was analyzed by ANOVA using Duncan's post-hoc test for comparisons among means at $p \leq 0.05$ when appropriate.

Results

Acetaminophen Liver Injury at 6, 24 and 48 hr

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 hr. 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 if changes could be found by image analysis of serum proteins separated by two dimensional gel electrophoresis for each animal. As expected, no detectable histopathologic changes were observed at 150 mg/kg acetaminophen nor were clinical chemistries altered from control (data not shown). However, notable histopathologic and serum protein changes were observed at the higher dose of 1500 mg/kg acetaminophen from 6 to 48 hr. Results in Figure 1 show representative histopathology at 6, 24 and 48 hr at the 1500 mg/kg acetaminophen dose. The associated numbers of serum protein changes at each time point are also presented in Figure 1 for the low (150 mg/kg) and high dose (1500 mg/kg) of acetaminophen. After 6 hr of 1500 mg/kg acetaminophen, hepatocytes in centrilobular areas showed some degenerative changes manifested by cytoplasmic microvacuoles in centrilobular hepatocytes in 4 of 5 total rats (4/5). At 24 hr, acetaminophen treatment produced marked centrilobular necrosis combined with sinusoidal erythrocytic congestion in all animals which declined by 48 hr at which time only 3/5 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/AST values of vehicle controls at 6 hr were 95/125 U/L, at 24hr were 91/126 U/L and 48 hr were 74/102 U/L. At 1500 mg/kg acetaminophen the ALT/AST values showed no change at 6hr, were 69X/86X-fold increased above time-matched controls at 24 hr, and were 25X/29X-fold above time-matched controls at 48 hr. 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 hr, 24 hr and 48 hr, 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 hr (Figure 1, bar graph). A dose of 150 mg/kg acetaminophen produced a discrete number of serum protein changes from control (2 proteins at 6 hr; 3 proteins at 24 hr; and none at 48 hr). While only one protein was altered at 6 hr, administration of 1500 mg/kg, acetaminophen significantly altered 68 serum proteins after 24 hr of which 23 proteins were $>5X$ fold change and 20 proteins were newly

detectable (i.e. not in controls) in serum after acetaminophen treatment. By 48 hr, the number of altered serum proteins declined to 17 proteins of which only 1 protein was >5X changed and only 2 proteins were newly found with treatment versus control. Therefore, two dimensional gel separation and image analysis detected minimal changes at 6 hr, the greatest number and magnitude of serum protein alterations at 24 hr with a decline in serum protein changes at 48 hr 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 (MSN) in the common coordinate (master) gel. Table 1 contains the fold change versus time-matched control of identified proteins (Uniprot No.) after various times for either the 150 mg/kg or 1500 mg/kg acetaminophen dose. For the 68 protein isoforms changed at 24 hr, 41 (60%) were identified by mass spectrometry. At 48 hr, 14 proteins (82%) of 17 altered proteins were identified. Figure 2, Panel A displays an electronic two dimensional gel image of total separated serum proteins at 24 hr 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, ApoE and others. Some proteins were not detectable in control (ND Ctl) but were newly found in serum after acetaminophen treatment such as arginosuccinate synthase, urocanase, glycine N-methyltransferase, acyl-coA dehydrogenase, ARP3, GSH synthetase and others. While a modest fold change (<5X) occurred in many proteins, acetaminophen effects were more pronounced (>5X) with an increase or decline of proteins in serum, such as β -actin, glutamate dehydrogenase, alanine aminotransferase, aldehyde 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; www.ebi.ac.uk/GOA/) of identified serum proteins in Table 1 represent cellular enzymes in protein degradation, intermediary metabolism, and carrier proteins that appear at higher serum levels during liver injury as well as the appearance of inflammatory, acute phase and coagulatory proteins. Bar graphs in Figure 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) while 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 hr were validated by western blot with available antibodies (Figure 3). Acetaminophen treated rats exhibited widespread necrosis and ALT (>4000 U/L) values were increased above control for acetaminophen rats (lanes 6, 8–10), although the ALT value for the rat in lane 7 was not as high (947 U/L). Immunodepleted serums 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 kD form (top blot) was most responsive to acetaminophen for 3 of 5 rats (lanes 8–10). The 160 kD form was not substantially affected by treatment (data not shown). Appearance of serum catalase and β -actin were apparent in 4 of 5 rats after 1500 mg/kg acetaminophen treatment (lanes 6, 8–10). Except for aminopeptidase, signals in control lanes 1–5 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 6–48 hr

experiment suggested that a 48 hr 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 five 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 followup analyses as well as histological evaluation.

Acetaminophen Liver Injury from 24 to 120 hr

In a second study, rats exposed to 1500 mg/kg acetaminophen showed signs of acute liver injury (Figure 4) and ALT/AST elevations after 24 hr as before. Evaluation of histopathology after acetaminophen at 24 hr showed centrilobular necrosis (mean score of 2.5 in 4/4 rats) with sinusoidal erythrocytic congestion that was diminished after 72 hr (mean necrosis score of 1.75 in 4/4 rats) which had resolved to no necrosis in any rats by 120 hr (data not shown). In addition, discrete areas of mineralization were also observed in liver sections at 72 and 120 hr after acetaminophen (data not shown). Figure 4 shows that the increased levels of ALT and AST levels observed after 24 hr of acetaminophen had greatly declined by 72 hr but peaks of serum bilirubin, bile acids and alkaline phosphatase occurred at this time. These serum changes suggested impairment of liver function at 72 hr after acetaminophen despite a decline in ALT/AST. However, by 120 hr serum clinical values had nearly returned to normal or slightly below normal for all serum indicators of liver function (Figure 4). Notably, no premature animal deaths occurred prior to 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 Figure 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 hr, but 4/5 rats were positive for catalase by 24 hr and all 5 rats showed the presence of catalase at 48 hr after 1500 mg/kg acetaminophen. In the second experiment, serum catalase was found in all rats after 24hr (3 prominently) but signal was reduced at 72 hr and was similar to controls by 120 hr. In the adjacent panel of Figure 5, mean serum catalase activities were consistent with western dot blotting results and were highest at 24–48 hr and declined by 72 hr and 120 hr.

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 6–48 hr and 24–120hr after acetaminophen. TNF α levels increased as early as 6 hr in the first experiment (Figure 6 left panel) and continued to rise at 24 and 48 hr after acetaminophen. In the second experiment, acetaminophen treatment increased serum TNF α concentration after 24hr but levels declined by 72 and 120 hr. Thus, the highest levels of serum TNF α were attained at 24 to 48 hr after 1500 mg/kg acetaminophen treatment.

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 6–48hr were incubated along with a blank membrane (no rat sera). Chemiluminescent detection was simultaneously performed with all membranes (7

membranes/experiment) for intercomparability among treatment groups in each experiment of 6–48hr or 24–120hr. Two serum factors, MCP-1 and TIMP-1, were found to be consistently increased above controls during acetaminophen treatment (Figure 7 A, B). Panel D of Figure 7 shows the capability of this antibody array method to detect serum cytokine and chemokine changes after acute in vivo exposure of rats to the potent inflammagen, lipopolysaccharide (LPS).

The findings from antibody arrays were confirmed for MCP-1 (Figure 7, Panel C) by quantitative ELISA using serum from each animal. ELISA results showed no change from control at 6 hr but substantial increases in serum MCP-1 concentrations were detected at 24 and 48 hr after acetaminophen treatment. In the second experiment in which rats were exposed to 1500 mg/kg acetaminophen at 24, 72 and 120 hr, the increases in MCP-1 concentration at 24 hr were pronounced but returned to just above control levels at 72 and 120 hr. These results suggest that the highest levels for serum MCP-1 were observed at 24 to 48 hr 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 densitometric features were reproducibly detected on each two dimensional gel for each animal. Serum proteins as measured by differential two dimensional gel electrophoresis appeared remarkably stable in the absence of liver injury since so few changes were observed from 6–48 hr at the lower dose of acetaminophen or at 6 hr 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 hr, the number of serum protein changes were reduced by four fold compared to those observed at 24hr, clinical serum chemistries had declined, and histologic 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 ANIT, respectively (Amacher et al., 2005). Similar to the current study, they noted that 1000 mg/kg acetaminophen increased serum 4-HPPD and of 19 altered serum proteins, these four agents increased Gc globulin and malic dehydrogenase and also decreased PRBP. 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 impacts the serum proteome. Genomic analysis has shown transcript changes are detectable at early times (15 min) (Ruepp et al., 2002) or sub-histological toxicity (Heinloth et al., 2004) although the complexity of cellular events often obscures precise mechanisms of toxicity. In our study,

proteomic analysis did not reveal early changes in the serum proteome despite indications that apparent critical events were occurring at 6 hr (i.e. TNF α) prior to the later appearances of elevated serum ALT, AST and necrosis. The detection of early predictors of liver injury in serum await more sensitive proteomic methodologies matched to the kinetic unfolding of critical events in liver injury. However, we did discover the appearance of several serum proteins during peak injury and recovery periods not previously reported. Increases in serum fetuin B isoforms and arginosuccinate synthase, decrease in AT-III and alterations in complement C3 isoforms were consistently observed at 24 and 48 hr. We found serum protein changes previously observed with other drug-induced necroses from hepatic cytoplasmic compartments including aminopeptidase (Musana et al., 2004), glutamate dehydrogenase (O'Brien et al., 2002), the urea cycle enzymes arginosuccinate synthase and ornithine carbamoyltransferase (OCT) (Ikemoto et al., 2001), malic enzyme (Zieve et al., 1985) and urocanase (Trip et al., 1973). Increased serum glycogen phosphorylase has been proposed as a marker of acute ischemic cardiovascular disease (Apple et al., 2005) but is now reported for acetaminophen toxicity.

The findings of increased serum proteins not normally associated with acetaminophen necrosis might also be interpreted as beneficial host responses for recovery from liver injury. Gc-globulin and hemopexin can respectively scavenge free actin and heme released from necrotic cells that might otherwise lead to multiple organ failure after acute liver damage (Lee and Galbraith, 1992; Tolosano and Altruda, 2002). The 2 to 6 fold increase in various isoforms of serum complement C3 and C4a may contribute to inflammation and liver regenerative processes after injury (Markiewski et al., 2004). Increased serum isoforms of the cystatin-like protein, fetuin-B, at 24 and 48 hr are consistent with elevated serum fetuin-B recently reported for a murine leukemia model (Bhat et al., 2005).

In the present study, two isoforms of catalase were increased by 10 fold in serum at 24 hr after acetaminophen toxicity. Validation of serum catalase by western blot and biochemistry at 24, 48 and 72 hr demonstrated that it was enzymatically most active at 24–48 hr at times corresponding to greatest liver injury. However, catalase was still present in serum at 72 hr 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. Prior studies report that cultured endothelia are better protected from hydrogen peroxide by the serum of normal human subjects that contain increasing amounts of catalase activity (Leff et al., 1991). Importantly, therapeutics with catalase-activity have been shown to prevent sinusoidal and hepatocellular 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 reactive 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 proteins 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 dimensional gels. We complemented our proteomics strategy with cytokine antibody arrays and ELISA's to survey further serum protein changes. Serum TNF α levels peaked at 24–48 hr after acetaminophen but remained increased above control up to 120 hr. Similarly, antibody array and ELISA data in our study showed a 24–48 hr peak for MCP-1 and TIMP-1 after acetaminophen in a manner that might reflect a coordination of reparative processes. Proteolytic degradation of the extracellular matrix (ECM) is essential for tissue remodeling in liver repair, involving a combination of matrix metalloproteinases (MMPs) and their specific tissue inhibitors or TIMPs (Rudolph et al., 1999). A major factor in liver regeneration is the release and activation of pro-HGF from ECM

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/MMP proteolytic axis that ultimately controls the HGF-mitogenic signaling pathway (Mohammed et al., 2005). Although the exact roles of inflammation 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 combined with the wide quantitative range and complexity of serum protein expression often require use of multiple expression platforms as used in this study. Equally important are pre-purification strategies such as immunodepletion to remove the abundant, less informative serum proteins and allow more facile detection of low concentration serum proteins 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 6–48 hr, and with a toxic dose at 1500 mg/kg acetaminophen at 6 hr. This apparent stability of the serum proteome is in part due to proteomic platform limits in sensitivity but 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 improve 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 damaged hepatocytes as well as actively secreted signaling peptides (cytokines and chemokines) from both resident nonparenchymal (Kupffer, stellate, 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 pharmacologic effects and injurious effects of therapeutic agents.

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Figure 1.

Histopathology and associated changes in protein expression after acute exposure to acetaminophen in male, F344 rats at 6, 24 and 48 hr. Fasted rats were exposed to 0, 150 and 1500 mg/kg of acetaminophen by oral gavage as described in Methods. Shown here are representative H&E sections from sections of the left liver lobe from 6, 24 and 48 hr at 1500 mg/kg acetaminophen and control at 24 hr. Histology at 150 mg/kg acetaminophen was comparable to controls. At 6 hr after 1500 mg/kg acetaminophen, rats showed multiple cytoplasmic vacuolations (arrow) as features of early hepatocyte degeneration. At 24 hr after 1500 mg/kg acetaminophen, extensive necrosis and sinusoidal hemorrhage were evident. Arrows indicate extensive necrosis and erythrocytic congestion. Cellular regeneration in centrilobular regions (arrows) was apparent by 48 hr after 1500 mg/kg acetaminophen. The number of serum protein changes ($p < 0.01$) after acetaminophen treatment are shown in the center bar graph. A comparison was made of each two dimensional gel spot density value in animals from 150 mg/kg and 1500 mg/kg acetaminophen groups to spot density values of time-matched controls. Each group at each time point contained five male F344 rats. Serum proteins were separated on two dimensional gels for each individual animal and spot densities were registered and calculated.

Figure 2.

Annotated two dimensional gel image and bar graph of individual protein responses after 24 hr acetaminophen exposure. Panel A shows the master two dimensional gel image of serum proteins at 24 hr after acetaminophen exposure. Sypro Orange stained two dimensional gels from serum of individual animals were scanned, processed and analyzed by the Kepler image analysis software package as described in Methods. A composite electronic image of differentially expressed proteins was generated by combining and averaging protein densities of two dimensional gels from control, 150 mg/kg and 1500 mg/kg acetaminophen treatment groups at 24 hr. The annotated image in Panel A shows a portion of identified proteins that were increased by 1500 mg/kg acetaminophen. Protein abbreviations used in Panel A are appear in parentheses below the full name in Table 1. In Panel B, the bar charts show the relative densities for selected proteins appearing in Panel A. Individual bars are the densities of the specific protein for each animal in the treatment group for the three groups, (C)ontrol, 150 mg/kg (a)cetaminophen, and 1500 mg/kg (A)cetaminophen group (n=5/group). For some animals, no protein could be detected so that no bar appears. Abbreviations in the bar chart are: aminopeptidase (AmPeptidase); ME-1 (malic enzyme-1); ALT (alanine aminotransferase); ALDH (aldehyde dehydrogenase-1 or ADH1); proteasα4 (proteosome subunit-α-type-4); GD (guanine deaminase); 4-OHPP Dioxy, (4-hydroxyphenylpyruvate dioxygenase); CA II, (carbonic anhydrase II); ARP-3 (actin-related-protein-3); AdHoCys'ase

(adenosylhomocysteinase); OCT, (ornithine carbamoyltransferase); AcCoA DH (Acyl-CoA dehydrogenase); GSH Synth (glutathione synthetase).

Figure 3. Western blot of differentially expressed proteins after 24 hr of 1500 mg/kg acetaminophen treatment. Each lane represents 20–30 μ g of serum protein after immunodepletion from individual animals of each group. The order of animals for each lane (control in lanes 1–5; acetaminophen in lanes 6–10) was maintained for each western blot.

Figure 4.

Clinical chemistry changes in protein expression after acute exposure to acetaminophen in male, F344 rats at 24, 72 and 120 hr. Fasted rats were exposed to 0 and 1500 mg/kg of acetaminophen by oral gavage. Male, F344 rats were treated with vehicle (0.25% CMC) or 1500 mg/kg acetaminophen by oral gavage and sacrificed after 24, 72 and 120 hr. Values represent the mean $\pm$ S.E.M. at n=4/group for serum clinical chemistries performed according to Methods. Abbreviations are ALT (alanine aminotransferase); AST (aspartate aminotransferase); ALP (alkaline phosphatase); Total or Direct Bil (Bilirubin); TBA (total bile acids); Units are IU/L (International Units) for ALT, AST and ALP; μ Mol/L for TBA; mg/dL for total and direct bilirubin. An asterisk (*) indicates statistical difference from time-matched control at p<0.05 by ANOVA and post-hoc test.

Figure 5.

Increased serum catalase and enzymatic activity during liver injury by acetaminophen. Data in Figure 1 and 3 suggested an association between the time of liver necrosis and appearance of catalase in serum after 1500 mg/kg acetaminophen at 24hr. Two separate studies were evaluated for serum catalase from 6–24 hr and 24–120 hr after vehicle control and 1500 mg/kg acetaminophen oral exposure using dot blot western and catalase activity. In dot blot westerns (left panel) the “–” indicates vehicle control and “+” is the acetaminophen treatment. Each dot represents serum from an individual rat. The bar charts to the right of the figure represent the mean $\pm$ SEM for catalase activity for control (grey bar) and acetaminophen (black bar) treatments. An asterisk (*) indicates statistical difference from time-matched control at $p < 0.05$ by ANOVA and post-hoc test.

Figure 6.

TFN α ELISA assay of serum from rats during liver injury from acetaminophen exposure. Two separate studies were evaluated for serum TNF α levels from 6–24 hr (left panel) and 24–120 hr (right panel) after vehicle control and 1500 mg/kg acetaminophen oral exposure. An asterisk (*) indicates statistically significant difference from time-matched controls at $p < 0.05$ by ANOVA and post-hoc test.

Figure 7.

Proteomic evaluation of rat serum by antibody array after liver injury by acetaminophen. An antibody array was used to screen for rat cytokine-chemokine expression during acetaminophen-induced liver injury from pooled rat sera from two separate studies from 6–24 hr (panel A) at $n=5/\text{group}$ and 24–124 hr (panel B) at $n=4/\text{group}$. Antibody array analysis was repeated twice for each experiment. Staining intensity by chemiluminescent detection was normalized by albumin positive controls (each blot contains 4 spots in upper left and 2 vertical spots in lower right). Open arrow ($\uparrow$) indicates MCP-1 and solid arrow ($\uparrow$) indicates TIMP-1. An ELISA assay (panel C) for rat MCP-1 was used to quantitate levels from individual rats for each treatment group (mean $\pm$ SE). An asterisk (*) indicates significant difference of acetaminophen treatment (solid bar) from time-matched vehicle control (grey bar) at $p<0.05$ by ANOVA and post-hoc test. Panel D shows a representative antibody array blot of rat serum during acute inflammation after 2 hr and 6 hr exposure to the inflammagen, LPS (5 mg/kg, i.p.) compared to saline control (10 mg/kg, i.p.). Pooled samples ($n=4/\text{group}$) were used for each blot in Panel D. Cytokines were detected by chemiluminescence. Note that Panel D contains four blots; a control blot each at 2 and 6 hr and an LPS blot each at 2 and 6 hr. Cytokines are defined under Abbreviations.

Table 1
Altered serum proteins with acetaminophen treatment in rats^a

MSN	Protein ID	Treatment/time 150 mg/ kg	1500 mg/ kg	Accession Number	GO Ontology ^b Number. Process/Function; Location	Fold Change
145	Fetuin-B		24h	Q9QX79	GO:0004869; Cys protease inhibitor, HGF, insulin regulation, inflam response; EC space	↑1.7
176			48h			↑1.8
223			48h			↑1.5
			24h			↑2.4
			48h			↑2.0
431	Gc Globulin (Vitamin D-binding protein)		24h	P04276	GO:0006810 Transport/ Actin, VitD binding; EC space	↑1.9
558			24h			↑2.4
228	Haptoglobin	6h		P06866	GO:0006508 Proteolysis/Hb binding; EC space	↓1.6
243			24h			↓14.4
289			48h			↑2.6
247	Complement C3 (C3)		24h	P01026	GO:0006956, GO: 0006954; Complement activation, Inflam response/ Endopeptidase inhibitor; EC space	↑5.8
			48h			↑4.0
267			48h			↓2.4
411			48h			↓1.9
839			48h			↑2.5
1395			6h			↓2.6
			24h			↓5.8
			48h			↓6.3
262	Antithrombin-III precursor (AT-III)		24h	P32261	GO:0007596; coagulation/ Ser endopeptidase Inhibitor, EC space	↓5.8
510			48h			↓1.9
265	Hemopexin	24h	24 h	P20059	GO:0006810; transport; EC space	↑2.1
365						↑1.8
296	Apolipoprotein H (ApoH)		24h	P26644	GO:0008201; heparin binding; EC space	↑1.9
323	Transthyretin (Trthy)	6h		P02770	GO:0006810; transport; EC space	↓2.5
			24h			↑6.4
337	Actin, cytoplasmic 1 (β-actin)		24h	P48975	GO:0030036; cytoskeleton, biogenesis/ cellular structure; cytoskeleton, cytoplasm	↑9.6
606			24h			↑15.7
399	Heat Shock Protein 70 (Hsc70)		24h	P19378	GO:0006457; protein folding; cellular	↑4.5
428	Apolipoprotein E (ApoE)		48h	P02650	GO:0042632, GO: 0000302; cholesterol metabolism, ROS response; EC region	↓1.5
538			48h			↓2.1
453	Aminopeptidase (APdase)		24h	Q9CPY7	GO:0019538; protein metabolism; cytosol	↑5.4
454	Plasma retinal-binding protein (PRBP)		24h	P04916	GO:0006810; transport; EC space	↓2.0
478	Glutamate dehydrogenase ^c , mitochondrial precursor (GDH)		24h	P10860	GO:0006520; AA metabolism; mitochondria	↑22.2
483	α-1-antitrypsin (α-1-AT)	24h		P17475	GO:0006953; acute phase response/ Ser endopeptidase inhib; EC space	↑1.9
546	Arginosuccinate synthase (ArgSuccSyn)		24h	P09034	GO:0006526, GO:0000050; Arg synthesis, urea cycle; cytosol	ND Ctl
			48h			
557	NADP-dependant malic enzyme (malic enzyme 1)		24h	P06801	GO:0006108; malate metabolism; cytosol	↑47.9
589	Complement C4A (Anaphylatoxin)	24h		P08649	GO:0006958, GO: 0006954; Complement activation, Inflam response/ Chemotaxis; EC space	↑2.7
		48h				↑2.3
612	Urocanase (urocanate hydratase)		24h	Q8VC12	GO:0006548; histidine catabolism/ lyase; cytoplasm	ND Ctl
625	Alanine Aminotransferase; (ALT)		24h	P25409	GO:0009058; biosynthesis/ Ala transaminase; cytoplasm	↑49.2
705	Glycine N-methyl-transferase ^c (GlyNMeTx)		48h	P13255	GO:0046500; SAM, FA metabolism/ methyl transferase; cytoplasm	ND Ctl
719	α-1-inhibitor III (α-1-Inh-III)		24h	P14046	GO:0006953; acute phase response/ protease inhibitor; EC space	↑2.0
745	Proteosome subunit α type 4 ^c		24h	Q9R1P0	GO:0006511; Ubiqu protein catabolism/ endopeptidase; cytosol	↑6.0

MSN	Protein ID	Treatment/time 150 mg/ kg	1500 mg/ kg	Accession Number	GO Ontology ^b Number. Process/Function; Location	Fold Change
777	Guanine deaminase (GD)		24h	Q9WTT6	GO:0006139; Nucleic acid metabolism/deaminase; cytoplasm	↑14.2
799	Delta-aminolevulinic acid dehydratase (ΔALADH)		24h	P06214	GO:0006783; heme synthesis/ metal binding; primarily cytoplasm	ND Ctl
780	Catalase		24h	P04762	GO:0042744; H2O2 catabolism/catalase activity; cytoplasm	↑10.7
937			24h		GO:0006629; lipid metabolism/Acyl CoA deHase; mitochondria	↑9.7
865	Acyl-CoA dehydrogenase long-chain specific, mitochondrial precursor (AcylCoA DH)		24h	P15650	GO:0005975; CHO metabolism/phosphorylase; cytoplasm	ND Ctl
887	Glycogen phosphorylase (GlyPase)		24h	NP595618	GO:006730; one-C metabolism/dehydratase; cytoplasm	↑165.1
965	Carbonic Anhydrase II ^c (CA-II)		24h	P27139	GO:0006520; GO: 0000050; AA metabolism, urea cycle; mitochondria	↑24.5
1001			24h		GO:0009072; Aromatic AA metabolism; hydrolase activity; primarily cytoplasm	ND Ctl
1093	Ornithine carbamoyltransferase (OCT) mitochondrial precursor		24h	P00481	GO:0006547; FTCD activity; golgi	↑107.6
1532			24h		GO:0006547; histidine metabolism/lyase; cytosol	ND Ctl
1196	Fumarylacetoacetase (FAA)		24h	P25093	GO:0006730; one-C metabolism/hydrolase; cytoplasm	ND Ctl
1306	Formimidoyltransferase- cyclodeaminase (FTCD)		24h	O88618	GO:0009072; aromatic AA metabolism/dioxygenase; cytoplasm	ND Ctl
1318	Histidase		24h	P21213	GO:0006928; cell motility/ structural molecule; cytoskeleton	ND Ctl
1483	Adenosylhomocysteinase (AHC)		24h	P10760	GO:0004887; GSH synthesis/ GSH synthesis; cytoplasm	ND Ctl
1486	4-hydroxyphenylpyruvate dioxygenase (4-HPPD)		24h	P32755	GO:0008152; metabolism/ dehydrogenase; mitochondria	ND Ctl
1526	Actin-related protein 3, mouse homolog; (ARP3)		24h	Q99JY9		
1540	Glutathione synthetase ^c (GSH Syn)		24h	P46413		
1542	Aldehyde dehydrogenase I, mitochondrial precursor ^c (ALD1)		24h	P11884		

^aDifferentially expressed serum proteins after APAP exposure in male rats found after proteomic analysis. Rats were exposed to 0 (vehicle), 150 and 1500 mg/kg of APAP for 6, 24 or 48 hr at n=5/group. Sera were collected for two dimensional gel separation and mass spectrometry analysis. Serum from each rat was immunodepleted of albumin, Ig's and transferrin prior to two dimensional gel electrophoresis of 150 µg serum protein. Image analysis determined differential protein expression among time-matched control and treated animals prior to protein identification (ID) by mass spectrometry according to Methods using the common name as referenced by the UniProt accession number (<http://www.pir.uniprot.org/>). Scanned images of all two dimensional gels were registered to a common x,y coordinate system to which proteins were assigned master spot numbers, abbreviated as MSN. In some cases, multiple protein isoforms were analyzed as the same protein identity by mass spectrometry which is indicated by different MSN's for the same protein. Altered protein levels are presented as increased (↑ arrow) or decreased (↓ arrow) fold changes at the p<0.01 level of statistical significance at the indicated times of 6, 24 or 48 hr. In the case of newly appearing proteins in serum from acetaminophen treated rats which were not detected in control rats (ND Ctl), no fold change was assigned.

^bGene ontology (<http://www.geneontology.org/>) or GO terms are included for key Biological Process(es); function and subcellular location are briefly described. GO numbers are listed for Biological Processes. Abbreviations in Table are in the order of appearance: Cys, cysteine; HGF, hepatic growth factor; inflam, inflammation; VitD, vitamin D; EC, extracellular; Hb, hemoglobin; Ser, serine; ROS, reactive oxygen species; AA, amino acid; Arg, arginine; deHase, dehydrogenase; inhib, inhibitor; Arg, arginine; Ala, alanine; FA, fatty acid; Ubiq, ubiquitin; H2O2, hydrogen peroxide; CHO, carbohydrate.

^cKnown acetaminophen adducts (James et al., 2003; Park et al., 2005)