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Gene Expression Profiling of Rat Livers Reveals Indicators of Potential Adverse Effects

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This study tested the hypothesis that gene expression profiling can reveal indicators of subtle injury to the liver induced by a low dose of a substance that does not cause overt toxicity as defined by conventional criteria of toxicology (e.g., abnormal clinical chemistry and histopathology). For the purpose of this study we defined this low dose as subtoxic, i.e., a dose that elicits effects which are below the detection of conventional toxicological parameters. Acetaminophen (APAP) was selected as a model hepatotoxicant because (1) considerable information exists concerning the mechanism of APAP hepatotoxicity that can occur following high doses, (2) intoxication with APAP is the leading cause of emergency room visits involving acute liver failure within the United States, and (3) conventional clinical markers have poor predictive value. Rats treated with a single dose of 0, 50, 150, or 1500 mg/kg APAP were examined at 6, 24, or 48 h after exposure for conventional toxicological parameters and for gene expression alterations. Patterns of gene expression were found which indicated cellular energy loss as a consequence of APAP toxicity. Elements of these patterns were apparent even after exposure to subtoxic doses. With increasing dose, the magnitude of changes increased and additional members of the same biological pathways were differentially expressed. The energy loss suggested by gene expression changes was confirmed at the 1500 mg/kg dose exposure by measuring ATP levels. Only by ultrastructural examination could any indication of toxicity be identified after exposure to a subtoxic dose of APAP and that was occasional mitochondrial damage. In conclusion, this study provides evidence that supports the hypothesis that gene expression profiling may be a sensitive means of identifying indicators of potential adverse effects in the absence of the occurrence of overt toxicity.

Key Words: **toxicogenomics; mitochondrial toxicity; hepatotoxicity; acetaminophen.**

To evaluate the adverse impact of exposures to xenobiotics on human health, indicators of biological response are needed that are sensitive, informative, and reproducible. The ideal biomarker should provide an indication of potential toxicity at times or doses preceding overt tissue damage, toxicity, or disease initiation. Many have speculated that this goal could be accomplished through genomic and proteomic approaches (Bandara *et al*., 2003; Bichsel *et al*., 2001; Tugwood *et al*., 2003).

One of the expectations of toxicogenomics, i.e., the incorporation of genomics into toxicology, is that global gene expression analysis will serve as "digital pathology," detecting subtle disturbances of biochemical pathways caused by low levels of an environmental stressor, which, if increased, will lead to serious cell and organ dysfunctions. We hypothesized that such changes in gene expression pattern could be predictive of toxicity if they reflect perturbations of vital cellular pathways. With increasing severity of the insult, the degree of disturbance of these pathways would be expected to increase, ultimately resulting in overt tissue damage as detected by traditional measurements of toxicity. The threshold between subtoxic adverse effects and toxic tissue injury would be marked by additional gene expression alterations representing pathways such as apoptosis, necrosis, or inflammation. In the studies described below, we define a toxic dose as any dose that causes either one or more of the following changes in the target organ: release of intracellular enzymes (e.g., lysosomal enzymes), histologically apparent cell death, or biochemically measurable organ dysfunction. We define as subtoxic a dose that causes none of these classic toxicologic effects, but induces changes in vital cell pathways, as manifested in expression changes of genes involved in such pathways.

To test our hypothesis we exposed rats to acetaminophen (APAP) ranging from doses expected to have no hepatotoxic effect to doses known to cause liver toxicity (Echard *et al*., 2001). APAP was selected as the test chemical for our studies because considerable information exists concerning its adverse effects in the liver. APAP is metabolized by cytochrome P450 isoforms to the highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which at therapeutic doses is detoxified by

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conjugation with glutathione (GSH; Mitchell *et al*., 1973). However at toxic doses of APAP, GSH is depleted and NAPQI covalently binds to cysteine residues on proteins (Pumford *et al*., 1997). These adducted proteins have been suggested to contribute to mitochondrial damage, inhibiting mitochondrial respiration and resulting in depletion of ATP (Pumford *et al*., 1997), which has been suggested to contribute to cell injury (Farber, 1973). In addition to the role of NAPQI, nitric oxide and its oxidation products may contribute to hepatic necrosis (Knight *et al*., 2002). APAP is one of the most widely used nonprescription analgesic and antipyretic drugs. Currently intoxication with APAP is the leading cause of drug related acute liver failure in the United States (Lee, 2003). The lethal toxicity in humans resulting from acute APAP overdosing is well documented and is currently a matter of significant health concern (Roberts and Morrow, 2001). The results of our studies suggest that subtle changes in expression patterns of vital cellular pathways induced by low doses of APAP may be indicative of overt toxicity occurring at higher doses.

MATERIALS AND METHODS

Chemical. Acetaminophen (APAP, 99% pure) was obtained from Sigma Chemical Company (St. Louis, MO) and suspension formulations prepared by mixing with 0.5% aqueous ethyl cellulose (USP/FCC grade; Fisher Scientific Company, St. Louis, MO).

Animals and animal care. Male F344/N rats, approximately 36 ± 3 days of age, were obtained from Taconic Laboratories, Inc. (Germantown, NY) and were approximately 89 ± 3 days of age when placed on test. The rats were housed three per cage in 22 in. (l) × 12.5 in. (w) × 8 in. (h) polycarbonate cages (Lab Products, Inc., Seaford, DE) with polyester cage filters (Snow Filtration Co., Cincinnati, OH). The room temperature was between 71 and 75°F, humidity between 36 and 48%. The rats had a 12-h light period from 0600–1800 h with a 12-h dark period from 1800–0600 h. Dosing occurred between 0900 and 1100 h. The animals had *ad libitum* access to irradiated NTP-2000 wafer feed (Ziegler Brothers, Gardners, PA) and *ad libitum* access to city water (Durham, NC).

Study design. For gene expression analysis, groups of three male rats each received a single dose of APAP by gavage at doses of 0 (vehicle only), 15, 50, 150, or 1500 mg/kg and were sacrificed after 6 (only sacrifice time for 15 mg/kg), 24, or 48 h. The entire study was replicated for a biological confirmation of results. Gene expression analysis was performed on both biological replicates although the data presented in this manuscript are derived primarily from one of the two biological replicates for clarity. For measurement of ATP content, two additional biological replicates with groups of two male rats each were dosed with 0 (vehicle only), 50, 150, or 1500 mg/kg APAP and sacrificed after 3, 6, 24, and 48 h. Experiments were performed according to the guidelines established in the NIH Guide for the Care and Use of Laboratory Animals (Council, 1996) and an approved Animal Study Protocol was on file prior to initiation of the study.

Necropsy procedures. Animals were euthanized with carbon dioxide from a regulated source 6, 24, or 48 h following dosing. Each treatment group had a concurrent vehicle-treated control group to minimize effects due to circadian rhythm. The necropsies took place within 1 h of the scheduled period of time. Blood for hematology and clinical chemistry was promptly drawn from the posterior vena cava. The right kidney and liver were weighed. A mid-sagittal section was taken from the left lateral lobe of the liver and the left kidney for histology, the remainder of the left lateral lobe quickly minced and frozen in liquid nitrogen for differential gene expression analysis. The remainder of the liver lobes and both kidneys were frozen in liquid nitrogen for future studies. The time from drawing of the blood sample to freezing of the liver for differential gene expression analysis was generally less than 90 s. Tissues were stored at −80°C until processed for RNA extraction.

For harvesting of the liver samples for measurement of ATP content, the animals were anaesthetized with pentobarbital (pentobarbital sodium injection, Abbott Laboratories, North Chicago, IL), (50 mg/animal, ip), after we observed in pilot studies ATP loss in liver samples from rats euthanized with carbon dioxide (data not shown). We utilized the cold clamp method as originally described by Easom and Zammit (1984). Briefly, a metal clamp was supercooled in liquid nitrogen and served as an *in situ* flash-freezing device. As demonstrated by Davies and colleagues (Davies *et al*., 1992) only liver samples frozen *in situ* were protected from artificial ATP loss and subsequent activation of AMPK.

Histopathology. After tissues were collected for differential gene expression, cross sections of the left, median, right anterior, right posterior, and caudate lobes were fixed for 24 to 48 h in 10% neutral buffered formalin. The tissues were embedded and routine H&E slides were made. A study pathologist (P.E.B.) initially evaluated all liver sections in a blind fashion. A second pathologist (G.A.B.) reviewed the diagnosis. Discrepancies between the pathologists were resolved by a Pathology Working Group review (Boorman and Eustis, 1986).

Ultrastructure analyses. Six additional rats were treated in an identical fashion to the previous studies, two animals each at doses of 50, 150, and 1500 mg/kg APAP, solely for ultrastructural analysis of livers at 6 h after dosing with two vehicle-gavaged rats serving as controls. The dosings were staggered to assure that each animal was sacrificed at 6 h ± 5 min. Rats were deeply anesthetized with nembutal, the portal vein exposed and catheterized, and the liver perfused for 30 min with 3% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.2. The left lateral lobe was minced into 1 mm cubes and held overnight refrigerated in 3% glutaraldehyde solution, post fixed in OsO_4, and processed further for electron microscopic evaluation. Centrilobular areas were identified on $\frac{1}{2}\mu$-thick sections stained with toluidine blue. Thin sections, approximately 80 nm, were then stained with uranyl acetate and lead citrate and examined using a Philips EM 400 electron microscope. When ultrastructural mitochondrial alterations were found in periveneous hepatocytes in rats dosed with 150 mg/kg, a second group was dosed and evaluated.

Clinical pathology. Hematology and clinical chemistry analyses were performed on all rats at study termination. Hematology analysis was performed on blood collected with EDTA as an anticoagulant. Erythrocyte, platelet, and leukocyte counts, hematocrit values, hemoglobin concentrations, mean cell volumes, mean cell hemoglobin, and mean cell hemoglobin concentrations were determined using an Ortho ELT-8 analyzer (Ortho Diagnostic Systems, Westwood, NJ). Clinical chemistry analyses were performed on serum samples using the Roche Cobas Fara chemistry analyzer (Roche Diagnostic Systems, Inc., Montclair, NJ). Analysis included blood urea nitrogen, creatinine, total protein, albumin, and total bile acid concentrations, and activities of alanine aminotransferase, alkaline phosphatase, creatine kinase, and sorbitol dehydrogenase.

RNA isolation. Total hepatic RNA was isolated from individual rat livers using QIAGEN RNeasy Maxi Kits® (QIAGEN, Valencia, CA) as previously described (Hamadeh *et al*., 2002). Equal amounts of RNA from each of three vehicle-only treated control animals at every dose and time period were pooled for control gene expression and compared with individual rats at each dose and time period. The samples were hybridized in duplicate with fluor reversal for each individual rat for a total of six microarray chips per dose and time period.

Microarray analysis. Rat clone cDNAs (Research Genetics, Huntsville, AL) (http://dir.niehs.nih.gov/microarray/chips.htm) were printed as previously described (Duggan *et al*., 1999; Hamadeh *et al*., 2002). The methods used to produce the chips are available at http://dir.niehs.nih.gov/microarray/methods.htm. The cDNA microarray analysis has been described in detail (Hamadeh *et al*., 2002). Fluorescent intensities were measured with an Agilent DNA Microarray scanner (Palo Alto, CA). To quantify and normalize the signal intensities from the cDNA spots on the image files, we utilized IPLabs

image-processing software (Scanalytics, Inc., Fairfax, VA) with the Array Suite 2.0 extension (National Human Genome Research Institute, NHGRI, Bethesda, MD; Chen *et al*., 2002).

We considered only genes that were differentially expressed at the 95% confidence level in both hybridizations per RNA pair. Using a binomial distribution (Casella and Berger, 1994) the probability of detecting a gene as differentially expressed by chance is $p < 0.0025$. To assure reproducibility of the observed gene expression changes between different individual animals, only genes that were determined to be differentially expressed in at least two out of three animals per biological replicate were included in further analysis (from the binomial distribution, the probability of this occurring by chance is <0.0001). Thus, scoring genes as differentially expressed at the 95% confidence level ($p = 0.05$) k = 2 times out of $n = 3$ biological replicates has a probability (P) of 0.0000187. We calculated the coefficient of variation (CV) for each gene across replicate hybridizations, using log2 ratio intensity values of the genes detected as differentially expressed at a given confidence level. Genes with a CV value greater than 0.8 were eliminated from further statistical analysis. Data was also subjected to analysis using a mixed linear model (Wolfinger *et al*., 2001) to identify significantly differentially expressed genes. This analysis utilized data from both biological replicates in order to gain the necessary statistical power.

Multiplex PCR. Twenty-five ng total RNA from each aliquot were reverse transcribed, followed by polymerase chain reaction, using the protocol established for eXpress Profiling multiplex RT-PCR (Johnson, 2002). PCR products were diluted in deionized water, mixed with GENESCAN 400HD [ROX], and run on an ABI 3100 capillary electrophoresis system according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Data was analyzed by Genescan for determination of product size and relative expression levels as defined by peak area. The resultant raw data was normalized against a control data point within the same reaction (β-glucuronidase).

Measurement of ATP content. Samples were prepared as described by Martin and McLean (1998). ATP was measured in the supernatant with an ATP assay kit (Calbiochem, San Diego, CA) according to the instructions of the manufacturer. We determined a linear range for this assay between 36 and 360 pmol ATP per reaction volume by performing dilutions of ATP solutions to generate standard curve measurements with every run of the assay (Suppl. Fig. 1). All test samples were diluted and measured within the linear range.

RESULTS

Rats were treated with a single dose of 0, 50, 150, or 1500 mg/kg of APAP by oral gavage. They were sacrificed at either 6 h after treatment, a time approximately 4 h after the expected peak in serum APAP levels (Roberts and Morrow, 2001), or 24 or 48 h after exposure.

Histopathology and Clinical Chemistry

Livers from animals receiving 50 or 150 mg/kg were morphologically normal and indistinguishable from control animals. At 24 and 48 h post dosing, animals receiving 1500 mg/kg showed a minimal to moderate degree of hepatocellular degeneration and/or necrosis. In addition, at 48 h there was minimal to moderate mitotic activity and mononuclear infiltrates (Fig. 1). No histopathological alterations were observed in the kidneys of any of the animals in the study.

Clinical chemistry parameters were not significantly altered at any time point in animals groups receiving 50 or 150 mg/kg APAP. At 24 and 48 h after administration of 1500 mg/kg, APAP induced marked increases in serum alanine aminotransferase, aspartate aminotransferase, sorbitol dehydrogenase and 5′-nucleotidase activities and total bile acid concentrations (Fig. 2 and Supplemental Table 1). In general, the increases were more prominent at 24 h and appeared to be ameliorating by 48 h. These increases in serum markers of liver injury seen at the high dose were in agreement with previous reports (Echard *et al*., 2001; Ruepp *et al*., 2002; Schiodt *et al*., 2002).

Based on the histopathological and clinical chemistry findings we concluded that 50 and 150 mg/kg doses of APAP produced no conventional signs of toxicity and thus would be considered subtoxic according to our criteria, while the 1500 mg/kg dose was clearly hepatotoxic.

Gene Expression Changes

In order to identify gene expression profiles indicative of APAP-induced effects, RNA was prepared from livers of control and treated rats and subjected to microarray analysis (Supplemental Fig. 2; complete data set available at: http://dir.niehs.nih.gov/microarray/datasets). Hierarchical clustering (Eisen *et al*., 1998) of all genes differentially regulated at any dose or time showed that there were clusters of genes similarly regulated at all three doses of APAP (Supplemental Fig. 3 and Supplemental Table 2). We were particularly interested in discovering gene expression changes after low doses of APAP that might be indicative of biological responses predictive of the observed toxic effects after the high dose. Review of differentially expressed genes after 50 and 150 mg/kg APAP revealed eight genes whose expression was also differentially regulated in response to the 1500 mg/kg dose. Additionally, the expression of an additional 29 genes was similarly changed in both the 150 mg/kg and the 1500 mg/kg dose groups (Supplemental Fig. 4).

Down Regulation of Genes Involved in Energy Consuming Biochemical Pathways

We found that transcript levels of enzymes involved in major energy consuming biochemical pathways were down regulated following administration of APAP, even after exposure to the lowest (50 mg/kg) dose (Table 1). These biochemical pathways include gluconeogenesis (glucose-6-phosphatase), fatty acid synthesis (fatty acid synthase; sterol-C4-methyl oxidase-like), cholesterol synthesis (3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1), and porphyrin synthesis (aminolevulinic acid synthase 1). After exposure to 150 mg/kg APAP, mRNA levels of additional enzymes involved in the same pathways were also repressed relative to levels in control livers (Table 1). These include genes involved in fatty acid synthesis (ATP citrate lyase) and cholesterol synthesis (farnesyl diphosphate synthase). In addition, genes involved in steroid synthesis (sulfotransferase hydroxysteroid gene 2), and the urea cycle (ornithine aminotransferase) were found to be down regulated following treatment with 150 mg/kg APAP. Analysis of differentially expressed genes after 1500 mg/kg revealed, among other

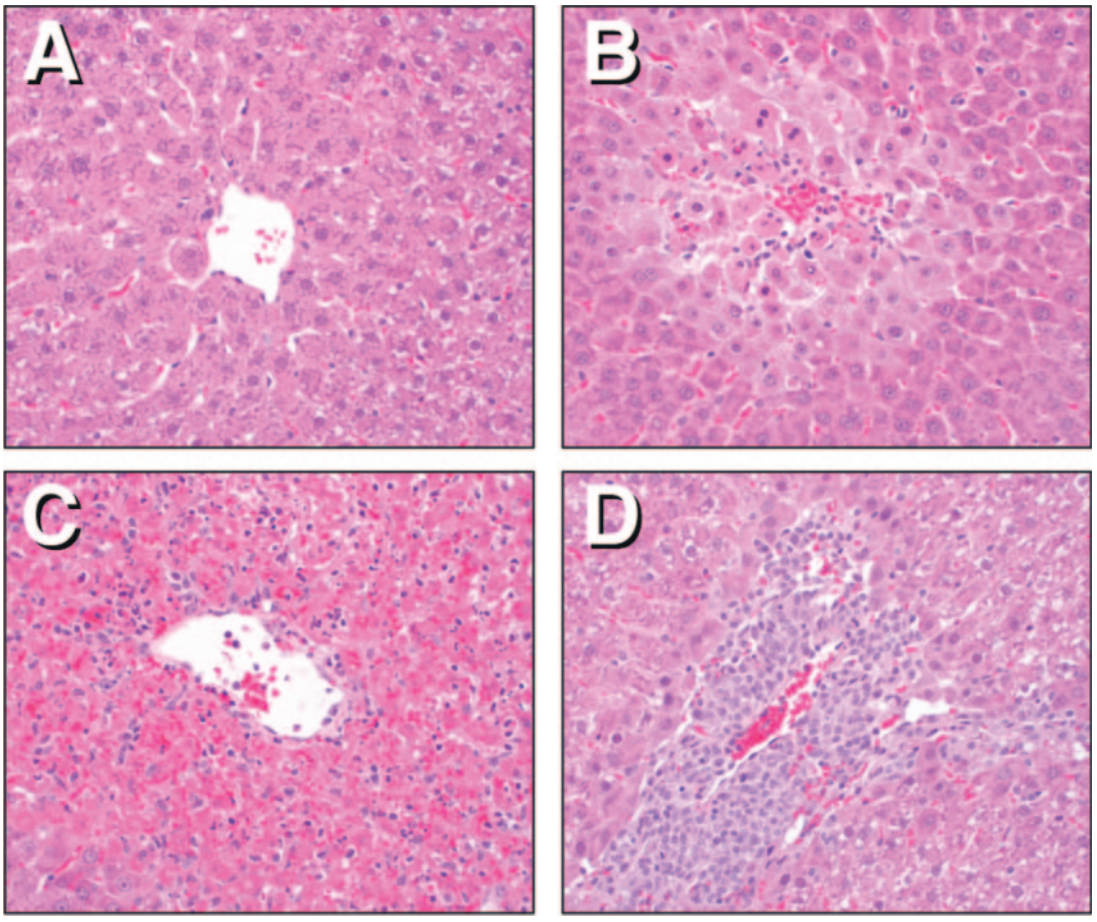

FIG. 1. Centrilobular necrosis in liver sections of animals that received 1500 mg/kg acetaminophen. Histological slides of rat livers, stained with H & E, of control animals (A) and animals exposed to 1500 mg/kg acetaminophen, sacrificed 24 (B) or 48 (C + and D) h after treatment. At 24 h after exposure cells bordering to the terminal hepatic venule appeared swollen and necrotic with inflammatory infiltrates. At 48 h post treatment in some centrilobular areas more extensive necrosis with hemorrhage is seen (C) while other centrilobular areas show loss of hepatocytes with dense mononuclear cell infiltrates (D).

responses, strong repression of transcript levels of multiple genes in all these energy demanding biochemical pathways (Table 1 and Fig. 3). We noticed that genes involved in microsomal (or peroxisomal) β-oxidation were down regulated following administration of 1500 mg/kg APAP (Table 1; enoyl coenzyme A hydratase 1; α-methylacyl-CoA racemase; isocitrate dehydrogenase 1, soluble). Since microsomal β-oxidation is not directly coupled to an energy producing electron transport system and the initial step in this pathway is an ATP requiring reaction, negative regulation of this pathway is consistent with the negative regulation of other energy consuming pathways. The differential expression of fatty acid synthase, farnesyl diphosphate synthase, sulfotransferase hydroxysteroid gene 2, and α-methylacyl-CoA racemase was confirmed by multiplex RT-PCR (Johnson, 2002). In summary we were able to observe down regulation of transcript levels of genes involved in energy requiring biochemical pathways after low, subtoxic doses of APAP, which cause neither pathological nor clinical chemistry changes. The degree of repression and the number of enzymes involved in each pathway increased as a function of APAP dose and corresponded with increased toxicity.

Up Regulation of Genes Involved in Energy Producing Biochemical Pathways

Analysis of differentially expressed genes after exposure to APAP revealed the induction of genes involved in energy producing biochemical pathways. The mRNA levels of several genes involved in both the control of glycolysis/gluconeogenesis (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1) and mitochondrial ω-hydroxylation (rat Cyp4a locus, encoding cytochrome p450 (IVA3) mRNA) were up regulated in response to 150 mg/kg APAP (Table 1 and Fig. 3). In addition, following treatment with 1500 mg/kg, indications of increased glycolysis (lactate dehydrogenase A; phosphofructokinase C), the tricarboxylic acid cycle (malate dehydrogenase-like enzyme), the pentose phosphate pathway (transaldolase 1; glucose-6-phosphate dehydrogenase; transketolase), and mitochondrial

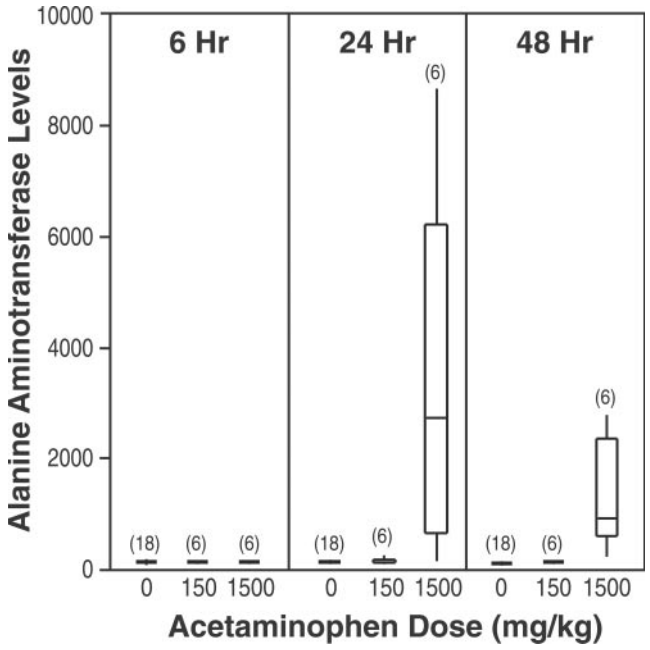

FIG. 2. Serum alanine aminotransferase elevated after exposure to 1500 mg/kg acetaminophen. Whisker graphs showing serum alanine aminotransferase levels in rats after exposure to acetaminophen. Data from control animals for the three different doses were combined in this visualization and contain therefore 18 rats per control group.

β-oxidation (acetyl-Coenzyme A acyltransferase 2/mitochondrial 3-oxoacyl-Coenzyme A thiolase; 2,4-dienoyl CoA reductase 1, mitochondrial) were observed. The differential expression of malate dehydrogenase was confirmed by multiplex RT-PCR (Johnson *et al*., 2002). These gene expression data provide evidence of an apparent attempt by liver cells to compensate for APAP-exposure effects by the production of energy, even following exposure to sub-toxic doses of 50 and 150 mg/kg APAP, doses that produce no overt classical evidence of toxicity. These changes are more pronounced in response to treatment with 1500 mg/kg APAP, where marked classical toxicity was observed.

Evidence of Ultrastructural Mitochondrial Damage in Livers Exposed to 150 mg/kg APAP

Gene expression data suggested a global energy loss starting at 50 mg/kg and increasing in severity with increasing doses of APAP and yet no evidence of cellular injury was apparent by conventional parameters of toxicity. Therefore, we performed ultrastructural examination of the liver tissue, with special attention to mitochondria, the energy producing units within the cell. We found swollen mitochondria with reduced opacity in centrilobular hepatocytes in rats exposed to 150 (Figure 4) and 1500 mg/kg APAP 6 h after treatment. These findings were confirmed in a second group of rats exposed to 150 mg/kg APAP. The mitochondrial changes were subtle, affected a small percentage of hepatocytes and were strictly limited to centrilobular areas. The affected hepatocytes were close to the central vein usually the first or second cell along the hepatic plate. While multiple centrilobular areas contained changes, other centrilobular areas within the same rat appeared unremarkable. However, occasional centrilobular hepatocytes with affected mitochondria were found in all rats exposed to 150 mg/kg APAP. No ultrastructural changes were found in controls or rats exposed to 50 mg/kg APAP.

ATP Depletion in Rat Livers after Exposure to APAP

Levels of total cellular ATP were measured in the livers of rats treated with 0, 50, 150, and 1500 mg/kg APAP at 6, 24, and 48 h following treatment. Reductions of total ATP were found to reach statistical significance only at 48 h after 1500 mg/kg (Table 2). No changes in ATP levels were observed at any time after treatment with 50 or 150 mg/kg APAP that were statistically significant. Since our gene expression data suggested an ATP depletion prior to 48 h, we examined livers at 3 h following APAP exposure and found that in fact ATP levels were significantly reduced at 3 h following exposure to 1500 mg/kg APAP (Table 2). Our data is in line with published results that measured comparable ATP levels in control animals (28.5 to 31.6 pmol/μg protein in this study, 13.5 ± 1.2 pmol/ μg protein in the study by Banerjee and colleagues [Banerjee *et al*., 1998]). It appears that this assay may lack the sensitivity necessary to detect the subtle changes in ATP levels suggested by the gene expression changes following exposure to doses of APAP lower than 1500 mg/kg.

Gene Expression Changes Suggestive of Stress

A protective role of metallothionein against APAP-induced oxidative damage has been reported (Liu *et al.*, 1999). We found that metallothionein mRNA levels were strongly up-regulated after 150 and 1500 mg/kg APAP. Using a pattern recognition algorithm (GeneSpring Version 5.0) we found nine genes with an expression pattern similar to that of metallothionein (minimum correlation = 0.95), as shown in the hierarchical cluster (Eisen *et al.*, 1998) in Figure 5. Besides two isoforms of metallothionein (metallothionein 3 and an EST highly similar to metallothionein-II), phospholipase C gamma 1 (PLC-γ1) was found to be very similar in its expression pattern across doses and time-points. Interestingly, at 6 h after 150 mg/kg APAP, expression changes within this cluster of 9 genes were similar to those seen after 1500 mg/kg at all three time points (Fig. 5).

DISCUSSION

In the present study, we investigated gene expression changes in rat livers in response to exposure to subtoxic and toxic doses of acetaminophen. Our results suggest that altered gene expression

TABLE 1
Differentially Expressed Genes Involved in the Regulation of Metabolic Pathways

		50 mg/kg APAP			150 mg/kg APAP			1500 mg/kg APAP		
Unigene acc. no.	Gene description	6 h	24 h	48 h	6 h	24 h	48 h	6 h	24 h	48 h
Gluconeogenesis										
AI043767	Ketohexokinase	0.99	0.95	1.02	0.96	1.05	1.05	0.90	0.74	**0.66**
AA964628	Glucose-6-phosphatase	0.69	**0.42**	0.78	1.4	**0.57**	1.07	1.23	**0.37**	**0.41**
AA964176	Glucokinase regulatory protein	0.99	1.04	1.02	0.95	1.06	1.05	**0.72**	**0.59**	**0.66**
AA924111	Glyceraldehyde-3-phosphate dehydrogenase	1.06	0.97	1.05	0.98	1.07	0.96	0.97	**1.80**	**2.09**
Glycolysis										
AA819266	Phosphofructokinase C	1.21	1.31	1.03	1.17	1.21	0.99	0.87	**2.55**	**2.67**
AI072330	Lactate dehydrogenase A	1.16	1.06	1.14	0.93	1.14	1.06	0.93	**1.49**	1.30
Control of glycolysis/gluconeogenesis										
AA997994	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1	1.10	1.01	0.89	0.96	**1.28**	1.12	1.25	0.97	0.80
Pentosephosphate pathway										
AA926108	Transaldolase 1	1.09	0.98	1.01	0.98	0.99	1.05	1.03	1.23	1.87
AA899102	Glucose-6-phosphate dehydrogenase	0.93	0.87	1.03	0.98	0.91	0.99	0.97	**1.75**	1.38
AA899042	Transketolase	0.99	0.87	1.04	0.94	0.92	1.09	0.92	**1.44**	1.29
TriCarboxylic acid (TCA) cycle										
AA900573	Malate dehydrogenase-like enzyme	0.99	0.90	1.04	1.02	1.01	1.03	1.20	1.33	1.60
Fatty acid synthesis										
AI137778	Fatty acid desaturase 1	0.91	1.19	1.05	0.98	0.84	1.07	0.57	**0.32**	0.82
AI043833	Stearoyl-Coenzyme A desaturase 1	0.89	0.87	0.95	0.93	1.20	1.24	1.11	0.68	**0.33**
AA956747	Delta-6 fatty acid desaturase	0.82	1.07	1.01	0.93	0.88	1.17	0.82	**0.54**	1.11
AA955881	Fatty acid synthase	0.78	**0.59**	0.97	1.05	**0.46**	0.94	0.43	**0.46**	1.15
AA859607	Sterol-C4-methyl oxidase-like	1.11	**0.71**	1.01	0.82	**0.49**	0.92	**0.54**	**0.59**	**1.54**
AA900486	ATP citrate lyase	0.75	0.74	1.06	0.92	**0.59**	1.03	0.73	**0.46**	1.14
Cholesterol synthesis										
AA955617	Farnesyltransferase beta subunit	0.92	1.02	1.00	0.95	1.03	1.05	0.94	**0.75**	1.01
AA924800	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	1.29	**0.76**	0.97	**0.73**	0.73	0.94	**0.40**	**0.42**	1.13
AA859192	Farnesyl diphosphate synthase	0.85	0.83	1.08	**0.72**	0.65	1.01	**0.69**	**0.56**	**1.76**
Steroid synthesis/processing										
AA923963	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	1.07	1.09	1.06	1.00	1.09	0.98	**0.63**	0.75	0.88
AA899367	17-beta hydroxysteroid dehydrogenase type 2	1.02	0.99	1.01	1.27	1.20	1.09	**0.65**	1.02	1.22
AA866493	Sulfotransferase family 1A, phenol-preferring, member 1	1.04	1.02	0.94	1.01	1.00	1.10	1.07	0.86	**0.67**
AA818024	Sulfotransferase Hydroxysteroid gene 2	1.10	1.13	0.98	0.85	1.02	**0.56**	**0.37**	**0.71**	0.99
β-oxidation-mitochondrial										
AA964573	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	1.13	0.97	1.11	1.12	1.17	1.03	1.06	1.77	1.46
AA875267	2,4-Dienoyl CoA reductase 1, mitochondrial	1.28	0.98	1.10	1.06	1.14	1.00	0.09	1.18	1.61
β-oxidation-peroxisomal										
AA926032	Enoyl coenzyme A hydratase 1	1.35	1.01	1.08	1.11	**1.21**	0.91	0.95	**0.76**	1.01
AA818115	α-methylacyl-CoA racemase	1.19	1.05	1.13	**1.47**	1.06	0.97	**0.33**	**0.53**	**0.54**
AA925731	Isocitrate dehydrogenase 1, soluble	1.08	0.98	1.05	1.00	0.98	0.99	**0.75**	**0.67**	0.99
AA818188	Acetyl-CoA acyltransferase, 3-oxo acyl-CoA thiolase A	1.05	0.96	1.03	1.14	1.04	0.99	0.88	0.89	**1.52**
Urea cycle										
AA818680	Ornithine aminotransferase	0.87	0.88	1.11	0.89	**0.66**	0.96	0.94	**0.51**	**0.29**
Porphyrin synthesis										
AA924489	Aminolevulinic acid synthase 1	1.45	0.96	**0.74**	1.05	0.87	1.34	1.37	1.45	0.80
AA874884	Heme oxygenase	1.06	1.01	0.99	1.05	0.93	1.02	2.05	**3.99**	**2.38**
Microsomal: ω-hydroxylation										
AA924591	Rat Cyp4a locus, encoding cytochrome p450 (IVA3) mRNA,	1.30	1.17	1.13	1.26	**1.62**	1.02	1.28	0.89	0.89

Note. Genes were identified as being significantly differentially expressed either at the 95% confidence level in at least two out of three animals in one biological replicate or by mixed linear model analysis of six animals from both biological replicate studies (Wolfinger *et al*., 2001). The numbers not in bold or underlined represent the fold change of each gene as an average of all three animals in one biological replicate after exposure to acetaminophen. **Bold** numbers indicate in which treatment group/time point the gene was identified as being differentially expressed. In these cases, the table presents the average fold change of those animals in which the respective gene was identified as being differentially expressed based on the 95% confidence level. Double underline indicates treatment group/time point that was identified as being statistically significantly differentially expressed when three animals from the original study were combined with three animals from the biological replicate study and were analyzed using Mixed Linear Model; the values shown in those cases represent the average fold change of all six animals.

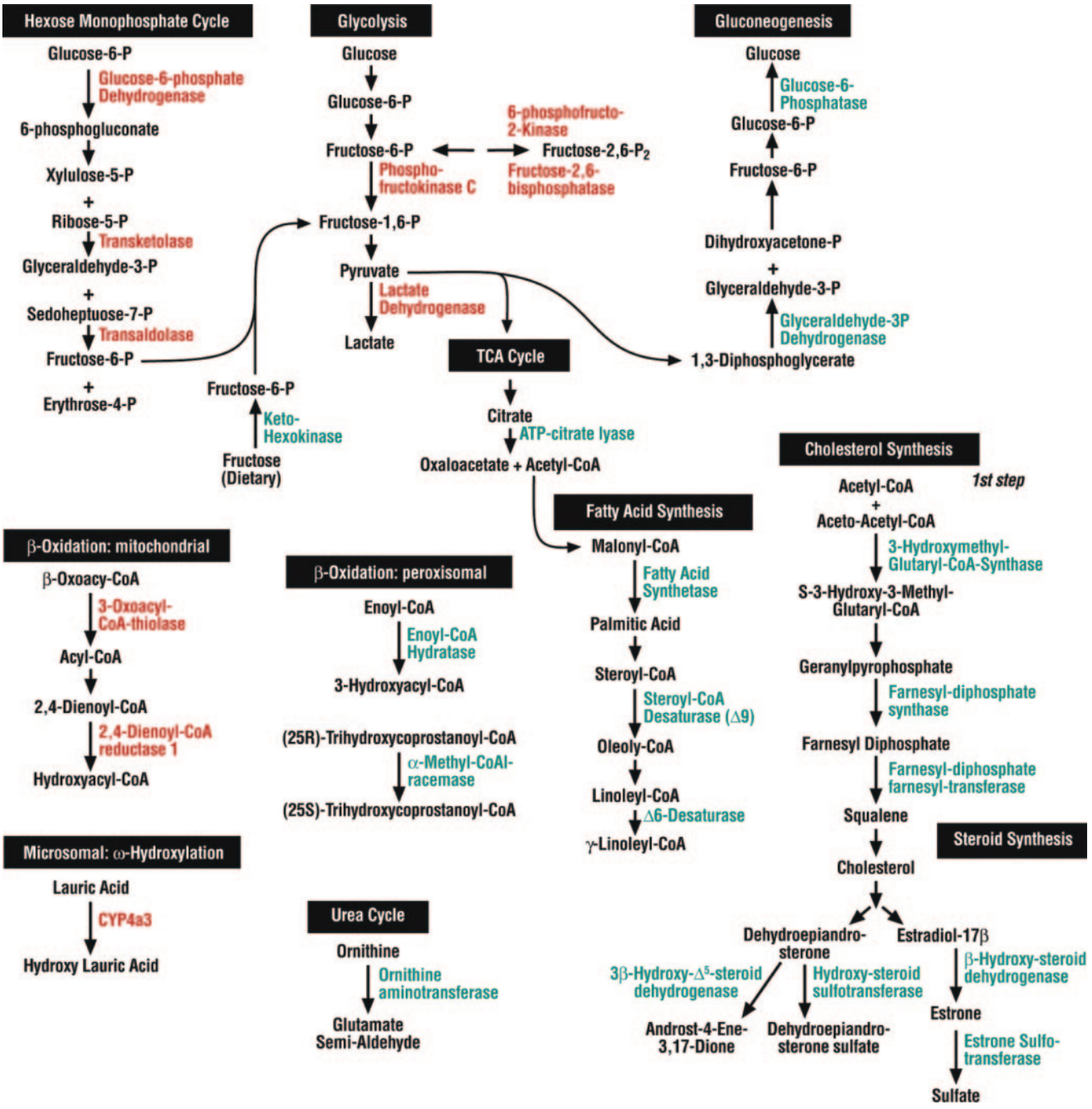

FIG. 3. Differentially expressed genes after exposure to APAP involved in metabolic pathways. Green indicates down regulated genes, red signifies up regulation.

patterns induced by exposure to a low, subtoxic dose of a potentially toxic agent may reveal signs of cell stress or subtle cell injury indicative of overt toxicity at higher doses. Thus, gene expression changes would appear to be more sensitive indicators of potential adverse effects than traditional measurements of toxicity. It should be pointed out that while the results of this study support our hypothesis, they do not allow for the unequivocal distinction between gene expression changes associated with adverse effects that are unique to APAP-induced liver toxicity, a more generalized liver toxicity effect, or some other liver effect. Additional studies are underway utilizing additional hepatotoxicants and nontoxic related compounds to address the specificity of these gene expression alterations as being truly indicative of adverse effects in the liver.

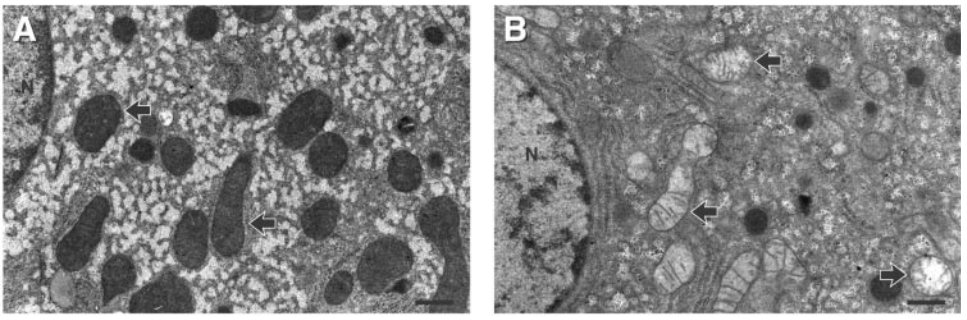

FIG. 4. Electron micrographs of centrilobular hepatocytes. (A) Centrilobular hepatocyte from control animal at 6 h after receiving the ethyl cellulose vehicle. (B) Rat exposed to 150 mg/kg APAP 6 h after treatment. Some of the mitochondria are swollen and less dense than the controls in a centrilobular hepatocyte. N indicates the nucleus, arrows point to mitochondria. (Magnification: 7100×)

TABLE 2
Effect of Exposure to APAP on Hepatic ATP Levels in Rats

Time	Control	50 mg/kg APAP	150 mg/kg APAP	1500 mg/kg APAP
3 h	31.1 ± 6.0	25.8 ± 5.1 (ns)	26.9 ± 3.0 (ns)	**14.5 ± 6.3***
6 h	28.6 ± 4.6	32.0 ± 9.0 (ns)	36.5 ± 9.2 (ns)	26.5 ± 5.1 (ns)
24 h	28.5 ± 1.9	30.5 ± 4.6 (ns)	33.8 ± 8.0 (ns)	22.8 ± 4.7 (ns)
48 h	29.5 ± 2.7	26.0 ± 5.0 (ns)	23.7 ± 3.0 (ns)	**18.4 ± 2.3***

Note. Results are expressed as pmol ATP/μg protein (mean ± SE). ns = not significant.

*Represents $p < 0.02$ for statistical differences between animals exposed to APAP and control animals.

Specifically, we identified changes in gene expression that may have the potential to serve as sensitive and predictive indicators of hepatotoxicty following exposure to APAP. These expression changes were observed even after subtoxic APAP doses. Livers from treated rats were examined at times that were selected to minimize gene expression changes related purely to the pharmacological effects of APAP while still allowing for detection of early responses associated with the adverse effects of the drug. We were able to identify gene expression changes indicative of cellular ATP depletion and stress in livers of rats exposed to subtoxic doses of APAP (50 and 150 mg/kg) and found that these signatures became more pronounced after treatment with an overtly toxic dose of APAP (1500 mg/kg).

Based on these results it appears that when the liver is stressed, even by subtoxic doses of APAP, a response is initiated to conserve energy by limiting energy consuming pathways and, at the same time, upregulating genes in pathways that increase ATP production and thus increase energy production. This was unexpected since 50 mg/kg APAP is well below the dose that produces overt toxicity as reflected by traditional clinical chemistry and histopathology parameters. In fact, no hepatotoxicity was observed with conventional measures of toxicity even after administration of a three-fold higher dose of 150 mg/kg APAP. Analysis of gene expression changes in the liver 6 h after administration of 15 mg/kg APAP revealed only three significantly differentially expressed genes (fatty acid binding protein 5, which was up regulated, and dual specificity phosphatase 6 and 17-beta hydroxysteroid dehydrogenase type 2, which were both down regulated), further supporting the dose dependency of the observed gene expression pattern.

Ultrastructural studies revealed damage to mitochondria after treatment with 150 mg/kg APAP consistent with the gene expression results. No mitochondrial alterations were observed by electron microscopy in livers exposed to 50 mg/kg APAP, even though genes involved in energy consuming pathways were down regulated at this dose. This suggests that such changes in gene expression are sensitive indicators of cellular stress before more drastic toxic effects become apparent. Evidence of APAP's mitochondrial toxicity has been previously reported (Bai *et al*., 2002; Immenschuh *et al*., 2000; Ruepp *et al*., 2002). For example Ruepp *et al*. (2002) found acute damage to mitochondria 2 h after ip injection of 150 mg/kg APAP into fasted mice. It is important to note that this is an overtly toxic dose in fasted mice in contrast to the same dose given by gavage to F344 rats fed ad libitum (Pessayre *et al*., 1980; Strubelt *et al*., 1981). Our earliest sampling for gene expression analysis was at 6 h. At this time severe changes in mitochondrial ultrastructure had occurred and evidence of impaired mitochondrial function was observed, as indicated by the down regulation of genes whose products are involved in key cellular processes which utilize ATP.

Besides effects on biochemical pathways of energy production and energy dependent pathways, we were able to detect a potential gene expression signature indicative of stress, involving nine genes, including metallothionein (see Fig. 5). Metallothionein, a key member of this gene cluster, is thought to be cyto-protective by serving as a zinc reservoir which functions as part of the hepatocyte antioxidant defense system (Haidara *et al*., 1999). Metallothionein induction has been reported in both rats and mice following APAP administration (Ruepp *et al*., 2002; Wormser and Calp, 1988). Another member of this gene cluster is PLC-γ1. It has been implicated in protective cellular mechanisms against oxidative stress (Bai *et al*., 2002), but has so far not been reported to be involved in the protective response against APAP toxicity. Several of the other genes responding like metallothionein were previously reported to be involved either in stress (Immenschuh *et al*., 2000; Nishino *et al*., 2002) or in

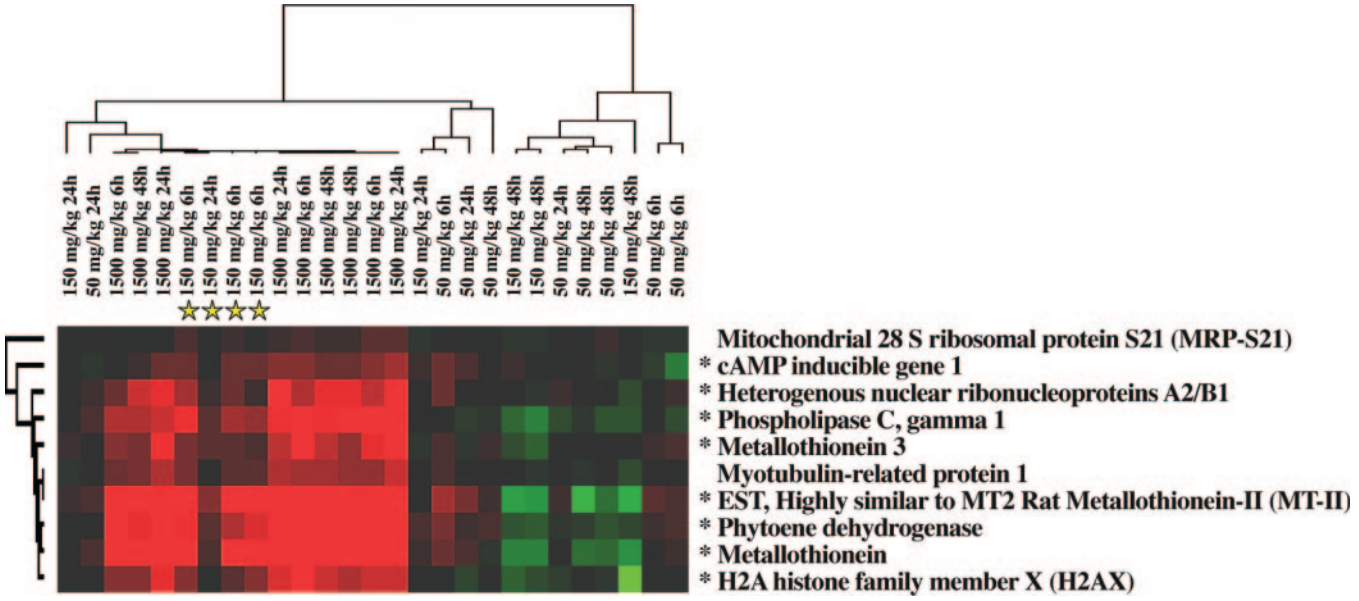

FIG. 5. Stress response genes similarly up-regulated after subtoxic and toxic doses of acetaminophen. Clustering tree of metallothionein and nine other genes that showed similar expression pattern (minimum correlation = 0.95) in a GeneSpring Trend analysis. Clustering analysis using the average $\log_2$ calibrated ratios for all 66 hybridizations in Eisen *et al*. (1998) Clustering (Cluster) and Visualization (TreeView) software, clustered with uncentered correlation coefficient and average inter-cluster linkage. *Indicates genes that have known implications in responses to stress or DNA damage.

DNA damage responses (Ford *et al*., 2002; Jackson, 2001; Petersen *et al*., 2001). However, two members of this group of genes, MRP-S21 and myotubulin-related protein 1, have not been previously reported to be involved in stress responses. Whether they are in fact stress response genes or are specifically related to APAP toxicity is presently unknown.

The observation that doses of acetaminophen considered to be nontoxic on the basis of traditional measures of toxicity cause changes in gene expression indicative of adverse cellular effects and cell stress responses has potentially important implications for safety assessment of drugs and environmental agents. Of course, not all alterations in gene expression should be viewed as deleterious. Careful analysis of the types of changes occurring is essential to distinguish between responses to potentially harmful effects and responses representing benign homeostatic adjustments. For instance, changes associated with a need to increase the capacity to metabolize and excrete a chemical would not be considered to be an adverse effect. However, we propose that changes in gene expression indicating cellular stress responses, and interruption or repression of vital cellular functions may serve as early markers of potential or impending toxicity, particularly when they increase in magnitude as a function of dose or duration of exposure. These changes would not themselves necessarily indicate toxicity but be predictive of potential adverse effects.

Investigations like the one presented here point to the complexity of delineating toxic effects, subtoxic/adverse effects and purely adaptive responses in the context of global genomic changes. The use of gene expression profiling in toxicology will make a critical reassessment of terms and definitions like toxicity, cellular injury, and cellular adaptation unavoidable. It is our hope that this report will contribute to stimulate this debate.

SUPPLEMENTAL DATA

Supplemental data are available at www.toxsci.oupjournals.org

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