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Expression Profiling of Acetaminophen Liver Toxicity in Mice Using Microarray Technology

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Drug-induced hepatotoxicity causes significant morbidity and mortality and is a major concern in drug development. This is due, in large part, to insufficient knowledge of the mechanism(s) of drug-induced liver injury. In order to address this problem, we have evaluated the modulation of gene expression within the livers of mice treated with a hepatotoxic dose of acetaminophen (APAP) using high-density oligonucleotide microarrays capable of determining the expression profile of >11,000 genes and expressed sequence tags (ESTs). Significant alterations in gene expression, both positive and negative, were noted within the livers of APAP-treated mice. APAP-induced toxicity affected numerous aspects of liver physiology causing, for instance, >twofold increased expression of genes that encode for growth arrest and cell cycle regulatory proteins, stress-induced proteins, the transcription factor LRG-21, suppressor of cytokine signaling (SOCS)-2-protein, and plasminogen activator inhibitor-1 (PAI-1). A number of these and other genes and ESTs were detectable within the liver only after APAP treatment suggesting their potential importance in propagating or preventing further toxicity. These data provide new directions for mechanistic studies that may lead to a better understanding of the molecular basis of drug-induced liver injury and, ultimately, to a more rational design of safer drugs.

Key Words: **acetaminophen; hepatotoxicity; liver injury; adverse drug reactions; drug toxicity; oligonucleotide microarrays; gene expression; toxicogenomics.**

Abbreviations used: ALT, alanine aminotransferase; APAP, acetaminophen; EST, expressed sequence tag; GADD, growth arrest and DNA damage inducible; HSP, heat shock protein; PAI, plasminogen activator inhibitor; PM, perfect match nucleotide sequence; MM, mismatch nucleotide sequence; OSIP, oxidative stress inducible protein; RT-PCR, reverse transcriptase-polymerase chain reaction; SOCS, suppressor of cytokine signaling.

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Drug-induced liver toxicity represents an important healthcare issue because it causes significant morbidity and mortality and can be extremely difficult to predict (1). Elucidating the mechanism(s) of drug-induced liver toxicity, therefore, is essential for the design of safer therapeutic agents. The antipyretic and analgesic drug, acetaminophen (APAP), has been the most widely studied of all drugs with hepatotoxic potential. It continues to be a significant cause of liver injury and lethality in humans (2). Studies have suggested that APAP-induced liver injury may be related to protein arylation (3), oxidative stress (4–6), disruption of calcium (7, 8) and mitochondrial homeostasis (9), alteration of transcription pathways (10–12), proinflammatory signaling (13–15), and the induction of cell death pathways (2). However, the precise molecular events occurring within the liver following APAP insult have still not been deciphered, and only a limited number of pathways involved in controlling toxicity progression have been identified (6, 16).

A more global approach is needed to elucidate the many factors that may play a role in the liver injury caused by APAP and other therapeutic drugs. Using an oligonucleotide microarray technique that is well suited for such a large-scale endeavor, we present an *in vivo* gene expression profiling approach for studying the mechanism(s) of APAP-induced hepatotoxicity. APAP was found to cause significant alterations in liver gene expression encompassing a wide array of physiological processes that include stress-responsiveness, cell cycling and cell growth, adhesion molecule expression, cytoskeletal and extracellular matrix organization, inflammatory responses, signaling pathways, and metabolic events. Many of the genes have not

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previously been reported and/or thoroughly investigated in the context of drug toxicity and, therefore, represent novel factors for further study of the mechanism(s) of APAP-induced liver injury. Similar factors may also play a role in the hepatotoxic potential of other drugs currently in use or in the developmental pipeline.

MATERIAL AND METHODS

In vivo treatment and tissue collection. C57B1/6 × 129/Ola hybrid mice (8 weeks; Taconic Farms) were acclimated for at least 6–7 days to a 12 h light/dark cycle in a humidity and temperature-controlled, specific-pathogen-free environment. This strain was selected because its genetic background corresponds with other strains used in our laboratory and in previous studies of APAP toxicity (6). Mice were allowed access to food and water until experimental use, whereupon food was withdrawn for 17–18 h before treatment (2, 3, 16). APAP (300 mg/kg) or saline (vehicle) was administered by intraperitoneal injection at 6:30 AM and food was restored after treatment. Six hours later, mice were sedated with CO_2, small blood samples were collected for serum enzyme analysis, and liver tissues were snap frozen in liquid nitrogen and stored at −80°C until use. All procedures were performed according to NIH guidelines for the care and use of laboratory animals.

Assessment of liver injury. Hepatotoxicity was assessed using both biochemical and histological techniques. Toxicity was initially evaluated by measuring serum alanine aminotransferase (ALT) activity using a miniaturized microtiter plate adaptation of a commercially available kit (Sigma). Liver sections taken at the same time as blood collection were processed by standard histological techniques and sections were stained with hematoxylin and eosin. All sections were later examined for histological evidence of liver damage.

RNA isolation. Total RNA was isolated from frozen liver tissues of individual mice using RNeasy Midi kits basically as described by the manufacturer (Qiagen). RNA concentrations and purity were determined spectrophotometrically and its quality further assessed by electrophoretic separation in 1.25% agarose gels and visualized with Vista Green nucleic acid dye (Amersham Pharmacia Biotech). Following these assessments, equivalent amounts of RNA from individual samples within each group were pooled to provide a representative sample for microarray analysis (APAP-treated, $n = 3$; Saline-treated, $n = 4$). Aliquots of RNA from individual animals were saved for subsequent confirmatory evaluations of gene expression.

Microarray protocol. Sample processing was performed exactly as described by the microarray manufacturer (Affymetrix) and outlined elsewhere (17, 18). Integrity of the final fragmented cRNA product was assessed using Test-2 Chips (Affymetrix) with the addition of manufacturer-recommended internal controls. Thereafter, expression analysis for each sample was performed using a set of two individual oligonucleotide microarrays (Mul1K sub A and sub B; Affymetrix) that can detect the expression of >11,000 known genes and expressed sequence tags (ESTs). Fluorescence intensities were analyzed using a laser confocal scanner (Hewlett Packard) and the accompanying GENE CHIP Analysis Suite (Affymetrix).

Expression analysis. Expression profiles were derived using the GENE CHIP 3.3 software (Affymetrix) and preestablished parameters. Software calculations, which compare the fluorescence intensity for each of the 20 perfect-matched (PM) and mismatched (MM) 25-mer oligonucleotide probe pairs designed for each known gene for EST (after background subtraction), provide the basis for a "decision" regarding the presence of that gene or EST in the sample (17). The "average differences" between each PM and MM (again after correction) provide a relative, but quantitative assessment of the gene or EST's expression. Furthermore, in order to provide data that is directly comparable between arrays, all data (including background) are scaled to a preestablished target intensity (1500) for analysis.

The effect of APAP on global gene expression in the liver was performed using the appropriately scaled saline-treated expression levels as baseline measurements. Genes or ESTs that were designated as being not significantly detectable above background signals ("absent" in the *Absolute Call* analysis), "not changed" in the comparison analysis, and altered less than 2.0-fold (*Difference Call*) were excluded from subsequent expression analyses. Thereafter, probe sets representing known genes and ESTs were grouped according to similarities in expression, fold changes compared to the saline-treated baseline, and reported physiological roles.

Reverse transcriptase-polymerase chain reaction (RT-PCR). APAP-induced alterations in liver expression of representative genes were confirmed using RT-PCR. RNA (1–2 μg) from individual mouse liver samples was reverse transcribed at 42°C for 30 min using self-contained Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech). After enzyme inactivation (95°C, 5 min), cDNA fragments were amplified for 20–36 cycles using gene-specific primers for c-fos, c-jun, and c-myc (all from Clontech), HSP27 (sense 5′-ATGACCGAGCGCCGCGTGC-3′; antisense 5′-TGGCTTCTACTTGGCTCCAGA-3′), HSP40 (sense 5′-TGGGCAAGGACTACTATCAGAC-3′; antisense 5′-CCGGGAAGATCACTTCAAAC-3′), HSP68 (sense 5′-AGAACGCGCTCGAATCCTAGT-3′; antisense 5′-TGGTACAGCCCACTGATGATG-3′); HSP70 (sense 5′-TGGAGATCATCGCCAACGACC-3′; antisense 5′-TCCTCCACGAAGTGGCTCACC-3′), HSP105 (sense 5′-AGAGTGAAGGTCAAAGTG-3′; antisense 5′-TTAAGAAGGTCTCTCCCT-3′), oxidative stress-induced protein (OSIP; sense 5′-AGATCCCAATGTCAATTTCC-3′; antisense 5′-GAGATGTGGGTATAGGGCAG-3′), CHOP-10 (sense 5′-CACATCCCAAAGCCCTCG-3′; antisense 5′-CTCAGTCCCCTCCTCAGC-3′), plasminogen activator inhibitor-1 (PAI-1; sense 5′-TCAGAGCAACAAGTTCAACTACACTGAG-3′; antisense 5′-CCCACTGTCAAGGCTCCATCACTTGCCCCA-3′), vascular cell adhesion molecule-1 (VCAM-1; sense 5′-CCCAAGGATCCAGAGATTCA-3′; antisense 5′-TAAGGTGAGGGTGGCATTTC-3′), LRG-21 (sense 5′-ATGATGCTTCAACATCAAGG-3′; antisense 5′-GCTGTTTCTCATTCTTCAGCTC-3′), and suppressor of cytokine signaling-2 (SOCS-2; sense 5′-GTTGCCGGAGGAACAGTCCC-3′; antisense 5′-ATGCTGCAGAGTGGGTGCTG-3′). Amplification of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH; sense 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′; antisense 5′-CATGTAGGCCATGAGGTCCACCAC-3′), served as a control for each sample. PCR products were resolved on 2% agarose gels in Tris-borate buffer and visualized using Vista Green nucleic acid gel stain.

RESULTS AND DISCUSSION

Recent technological developments in genomics and proteomics have dramatically increased the ability to study complex pathological events, including episodes of drug toxicity. The discovery of a key protein involved in cyclosporine A nephrotoxicity using a proteomics approach represents one such example (19). In order to facilitate the discovery of factors and pathways with a potentially important role in drug-induced liver injury, many of which may have escaped detection using traditional approaches, we initiated a global analysis of mRNA expression 6 h after a hepatotoxic dose of APAP. The selection of a time point during the early phase of toxicity was designed to facilitate investigation of genes with a role in the initiation and propagation of toxicity as well as those possibly involved in self-limiting injury progression.

TABLE 1

APAP-Induced Hepatotoxicity Significantly Alters Gene Expression in Mouse Liver Tissue within 6 h of Drug Administration[a]

Fold change	Number of probe sets ⇑	⇓
2.0–2.9	100	73
3.0–4.9	68	26
5.0–9.9	33	6
10.0–19.9	16	0
>20.0	9	1
Total	226	106

[a] The results represent numbers of probe sets (representing known genes and ESTs) in which APAP caused a minimum of 2.0-fold change in the expression parameters compared to the saline-treated baseline. Data in which the *Absolute Call* of a probe set was "absent" and where the *Difference Call* was "no change" were excluded from the analyses (see Materials and Methods).

A single dose of APAP (300 mg/kg, i.p.) to food-deprived C57B1/6 × 129/Ola mice caused a significant hepatotoxic response. Serum levels of ALT increased from 4.1 ± 2.5 IU/L (mean ± SD) in saline-treated controls ($n = 4$) to 849 ± 695 IU/L ($n = 3$) within 6 h of drug administration. Histological evaluation revealed an APAP-induced development of perivenous liver injury in each of the drug-treated animals (data not shown) corroborating many previous reports.

Using high-density oligonucleotide microarrays, the expression levels of >11,000 genes and ESTs were analyzed in these liver tissues. The oligonucleotide microarray technology available from Affymetrix has previously been shown to yield reproducible and quantitative results (18). Potential inter-individual variability was addressed by pooling RNA samples from individual mice within each group to yield a representative sample for analysis. Moreover, we selected a twofold threshold as a cutoff for significance, as has been reported by others (20–22), because smaller changes might be attributable to nonspecific hybridizations and/or biological variability. Whereas the overall pattern of liver gene expression was similar between APAP- and saline-treated mice (data not shown), the data revealed up-regulation and down-regulation of a significant number of genes over a very broad range of expression levels in response to APAP (Table 1). Gene induction appeared to be the more prevalent process, particularly as the level of expression changes increased (Table 1).

Analysis of the differential expression profiles indicated that APAP toxicity caused modifications in a multitude of physiological processes (Table 2). Among the subset of induced genes were a number of stress proteins and early response factors such as c-fos and c-jun, all of which have been investigated in recent studies of APAP toxicity (6, 16, 23–25). However, our analyses indicated that APAP toxicity elicited a wide array of cellular responses, much broader than earlier studies (6, 16, 24, 25) and more extensive and divergent than that reported in a proteomic study of APAP-induced liver injury in mice (23). Expression profiles suggested that APAP-induced liver injury resulted in the altered expression of numerous genes that encode for cytoprotective stress proteins, cell cycling and cell growth inhibitors, cytoskeletal components, adhesion molecules, extracellular matrix proteins, inflammatory mediators, a variety of early response factors and cell signaling molecules, cell death-related proteins, as well as many proteins involved in cell metabolism and those with a variety of other functions (Table 2).

Although the congruencies of our data with results from previous investigations (6, 16, 24, 25) support the validity of our microarray results, we confirmed the expression changes of a number of genes by RT-PCR using RNA from each mouse liver within a given treatment group. Despite some minor differences in the magnitude of change, the results were consistent with our microarray data (Fig. 1). Expression changes determined by microarray analyses (Table 2) correlated with APAP-induced alterations revealed in RT-PCR studies of individual samples within each group (data not shown), even at relatively low (e.g., 2.2-fold, SOCS-2) levels of change (Fig. 1). Moreover, a preliminary study using smaller scale, cDNA microarrays to analyze gene expression patterns within the liver of C57BL/6 × 129 F1 mice, 4 h following 300 mg/kg APAP (i.p.), confirmed the APAP-induced altered expression of a number of genes (Table 1; MB, *et al.*, unpublished data). Together, these data support the utility of the present oligonucleotide microarray analyses in drug-induced liver toxicity studies.

While it is beyond the scope of this report to highlight the potential significance of each altered gene, a number are of unique interest and therefore warrant specific mention: (1) Comparable to previous studies (24, 25), our analysis demonstrated the induction of early response genes following APAP. Microarray data also showed the induction of additional genes that inhibit cell cycling and arrest growth (Table 2). Growth arrest and DNA damage inducible protein (GADD) 45 and GADD 153 (i.e., CHOP-10) and growth arrest proteins (gas)-2 and 5 were dramatically induced within 6 h of APAP treatment (Table 2; Fig. 1). Depending upon the type of stress (26), GADD 153/CHOP-10 has been shown to halt cell growth in combination with other factors such as p21 and C/EBP, also increased following APAP (Table 2). A stoppage in cell growth/cycling during drug toxicity might allow cells to make a "decision" regarding their response to a drug insult. Increased expression of CHOP-10 (26) and gas molecules (27) has also been associated with the induction of apoptosis, possibly secondary to endoplasmic reticu-

TABLE 2

Functional Cluster Analysis of APAP-Induced Alterations in Gene Expression[a]

	Entrez definition or sequence similarity	GenBank or SwissProt ID	Fold change[b]
	Stress-responsive		
1	Chaperonin 10 mRNA	u09659	2.0
2	Apg-2 mRNA	D85904	2.2
3	Heat shock protein (HSP) 90-α (HSP86)[c]	P07900	2.2
4	DNAJ protein	P35515	2.3
5	DNAJ protein homolog 2	P31689	2.4
6	HSP86 mRNA	U88327	2.9
7	Actin polymerization inhibitor (HSP25-like)	Q00649	3.0
8	Adrenomedullin precursor mRNA[d]	u77630	3.1
9	Heat shock cognate protein HSC90-β	Q04619	3.2
10	Heat shock cognate 71 kD protein	P11142	3.2
11	Oxidative stress-induced protein (OSIP) mRNA	u40930	3.6
12	Metallothionein II gene	K02236	4.1[e]
13	Metallothionein I gene	V00835	4.7
14	Small HSP 25	l07577	5.0
15	Heme Oxygenase-1/Tumor-inducing p32kD	AA710574	5.7
16	Glutathione S-transferase Ya subunit gene		6.4
17	HSP105	d67016	8.3
18	Arsenite-inducible RNA-associated protein	aa044543	11.3
19	HSP 27 kD	P14602	13.3
20	Tumor-induced 32 kD protein (p32) mRNA	m33203	22.9
21	HSP68 mRNA	m12572	29.2
22	DNAJ protein homolog 1 (human; similar to HSP40)	aa542147	85.3
	Cell cycling and growth inhibition		
1	Gut-enriched Kruppel-like factor GKLF mRNA	u20344	2.2
2	TDD5 mRNA	U52073	2.9
3	Growth arrest specific (gas2) protein mRNA	M21828	3.1
4	FISP-12 protein mRNA	m70642	4.1
5	c-myc gene exon 3	l00039	5.5[e]
6	gas 5 mRNA		6.5
7	p21 (Waf1) mRNA	u09507	6.5[e]
8	GADD45 mRNA	l28177	9.0
9	GADD45 (Growth arrest and DNA damage inducible)	P24522	10.5
10	Growth factor-inducible immediate early gene (3CH134)	X61940	12[e]
11	GADD153	P65639	13.1
12	TIS21 gene	M64292	13.5
13	Growth factor-inducible protein (pip92) mRNA	m59821	22.1[e]
14	CHOP-10 mRNA	X67083	68.2[e]
	Adhesion and structural components		
1	Vascular cell adhesion molecule 1 (VCAM-1) mRNA	x67783	2.2
2	Tubulin beta chain	P02556	2.5
3	Endothelial cell activated protein C receptor mRNA	l39017	2.9
4	Actin I	P10982	2.8
5	α-1 type IV collagen mRNA	j04694	3.2
6	Entactin	X14194	3.2
7	Cytoskeletal gamma-actin mRNA	M21495	3.3
8	Secretory protein-containing thrombospondin motifs	D67076	4.7
9	Cysteine-rich protein 61 (Cyr61) mRNA	m32490	5.0[e]
	Inflammatory		
1	Corticosteroid binding globulin	X70533	−3.8
2	Shp gene		−2.3
3	C-10-like chemokine mRNA	u19482	−2.2
4	TNF-inducible protein TSG-6 precursor		2.7
5	L6 antigen mRNA	l15443	2.8
6	Suppressor of cytokine signaling-2 (SOCS-2) mRNA	U88327	3.0
7	T-lymphocyte activated protein (CHX-induced); immediate early response 2 protein	P17950	3.4[e]
8	Lymphocytic antigen 68/Cell surface antigen AA4	aa185911	3.8
9	Vascular endothelial growth factor mRNA	M95200	5.2
10	Ctla-2-α mRNA	X15591	6.4
11	Myeloid differentiation primary response mRNA encoding MyD116 protein	X51829	8.9[e]
12	Plasminogen activator inhibitor (PAI)-1 mRNA	M33960	14.1
13	Interferon (IFN)-related protein PC4	P19182	17.1[e]
14	INF β (type 2) mRNA	v00756	17.9

TABLE 2—*Continued*

	Entrez definition or sequence similarity	GenBank or SwissProt ID	Fold change[b]
	Cell signaling		
1	Retinally abundant regulator of G-protein signaling	U94828	−2.5
2	PP5/TFPI-2	D50586	−2.2
3	Serine-threonine protein kinase	X70764	2.0
4	Protein tyrosine phophatase (PRL-1) mRNA	U84411	2.0[e]
5	TI-225 mRNA	D50527	2.1
6	mATF4 (mTR67) mRNA	m94087	2.1
7	N10 gene for a nuclear hormonal binding receptor	x16995	2.1
8	JUN-D protein gene		2.3
9	NFIL3/E4BP4 transcription factor mRNA	U83148	3.1
10	Serine-threonine-tyrosine kinase mRNA	m38381	3.3
11	Transcription factor JUN-D	P15066	3.8
12	fosB mRNA	x14897	4.4[e]
13	TRA1 mRNA	d78354	4.7
14	C/EBP δ	X61800	5.6
15	Transcription factor AP-1 (proto-oncogene c-JUN)	P18870	16.6
16	Transcription factor LRG-21 mRNA	u19118	35.2
17	c-fos oncogene	v00727	37.4
18	Protein homologous to human c-Jun	x12761	41.2
	Death pathways		
1	RCK mRNA	D50494	2.2
2	Apoptosis inhibitor bcl-x gene, exon 3	u78031	2.6
3	TDAG51 mRNA	U44088	4.4
	Cell metabolism		
1	Antizyme inhibitor mRNA	AF032128	−122.3
2	Farnesyl pyrophosphate (FPP) synthetase	P05369	−5.9
3	Acetyl CoA synthase		−4.8
4	Squalene synthase mRNA	D29016	−4.2
5	Rat Oxidosqualene cyclase	aa670835	−4.0
6	3-β-hydroxysteroid dehydrogenase mRNA	M75886	−3.7
7	3-ketosteroid reductase mRNA	L41519	−3.6
8	Ornithine aminotransferase	X64837	−3.0[e]
9	Flavin monoxygenase 1 mRNA	U87456	−2.9
10	NAD-dependent 12-hydroxyprostaglandin dehydrogenase	U44389	−2.7
11	Glycerol-3-phosphate acetyltransferase	m77003	−2.2
12	Estradiol 17-β-dehydrogenase	D45850	−2.0
13	Spermidine/spermine N1-acetyltransferase mRNA	L10244	5.9
	Miscellaneous		
1	Amyloid A protein mRNA	u02554	−6.1
2	α-1 acid glycoprotein mRNA	m27009	−4.9
3	Retinoid X-receptor interacting protein mRNA		−4.7
4	Rat Calcium binding protein	aa203744	−3.1[e]
5	Sensescence marker protein 30 gene		−2.6
6	Liver-type glucose transporter	AA238219	−2.5
7	Apolipoprotein A-IV gene	m13966	2.4
8	Cathepsin L precursor		2.4
9	Calcineurin inhibitor	w91146	3.3
10	Insulin-like growth factor binding protein-1		4.3[e]
11	Zinc finger protein 216 (Zfp216)	aa510137	4.8
12	Glvr-1 mRNA	M73693	5.1[e]
13	Loricrin mRNA	m34398	7.5

[a] Following the exclusion of genes or ESTs whose expression was judged as being "absent" or altered by less than 2.0-fold, data were grouped according to published reports of the putative function of each gene product. Note, however, that the groupings are not mutually exclusive and do not include every single gene represented in Table 1. The classifications were designed instead to suggest cellular processes that seem to be modified by APAP-induced liver injury.

[b] Change in gene expression compared to that of baseline levels of saline-treated mice.

[c] Abbreviations: GADD, growth arrest and DNA damage-inducible protein; gas, growth arrest specific protein; HSP, heat shock protein; IFN, interferon; TNF, tumor necrosis factor.

[d] Shaded areas indicate that expression was detectable in the livers of APAP-treated mice alone, not in those of saline-treated mice, suggesting its induction by APAP treatment.

[e] Indicates genes or ESTs whose expression was altered similarly in a preliminary study using smaller scale, cDNA microarrays to analyze gene expression patterns within the liver of C57BL/6 × 129 F1 mice, 4 h following 300 mg/kg APAP (i.p.) (see Results and Discussion).

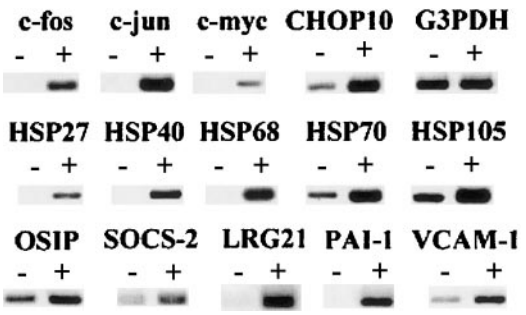

FIG. 1. Confirmation of microarray-determined alterations in gene expression by RT-PCR. Results shown represent pooled PCR reactions of 3–4 individually amplified and analyzed samples per group, each individual sample showing comparable results. Abbreviations: CHOP10, also called GADD153; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GADD, growth arrest and DNA damage inducible; HSP, heat shock protein; HSP68, inducible HSP70; HSP70, constitutive HSP70; OSIP, oxidative stress inducible protein; PAI, plasminogen activator inhibitor; SOCS, suppressor of cytokine signaling; VCAM-1, vascular cell adhesion molecule.

lum dysfunction (28), suggesting that they may play a role in APAP toxicity mediated through apoptotic mechanisms (2). (2) Oxidative stress inducible protein (OSIP; also called A170 and STAP) is induced by reactive oxygen species (ROS) and electrophiles in cells within the brain (29) as well as in macrophages and other immune cells (30, 31). We now show that it is induced during APAP-induced liver injury (Table 2; Fig. 1), possibly due to a combination of electrophilic drug metabolites and drug-induced ROS. Its inducibility by oxidative stress processes may be related to transcriptional activation by AP1 and NFκB (31), both of which are altered during APAP toxicity (see Table 2) (10, 24). Another recent study argued instead that OSIP is coordinately regulated with other stress-inducible genes including glutathione *S*-transferase Ya and heme oxygenase-1 (Table 2) by the transcription factor Nrf2 (32). Additional evidence suggests that a structurally similar human form of OSIP affects tyrosine kinases involved in T cell activation (30, 33). The possibility exists then that its induction during drug toxicity in the liver might impact both general stress responses as well as immunological signaling cascades, though the signaling mechanisms for these effects have not yet been fully elucidated. (3) Our expression analyses demonstrated a wide-ranging induction of stress proteins following APAP treatment (Table 2), including HSP27 (HSP25), HSP40, HSP70, HSP90α (HSP86), HSP90β, and HSP105, a considerable expansion upon those reported in an earlier protein expression study (16). However, little attention has been directed toward understanding the mechanistic implications of their induction in the context of drug toxicity. Aside from their generalized function as molecular chaperones, HSPs play important roles in signal transduction, inter-organellar transport within cells, cell cycling and proliferation, as well as anti-apoptotic responses (34). HSPs also possess immunomodulatory effects (35, 36) that may induce immunological responsiveness or tolerance. Therefore, the repertoire, extent, and subcellular localization of HSP induction, as well as their associated protein fragments, may have implications for immune-mediated and nonimmune-mediated drug reactions in the liver, hypotheses that have yet to be experimentally tested. (4) LRG-21 is a transcription factor believed to be involved in macrophage activation (37). It possesses c-fos and c-jun-like properties and is inducible by lipopolysaccharide, interferon gamma, and interleukin 4 (37). Our data also indicate its induction during APAP toxicity (Table 2; Fig. 1), possibly as a result of increased cytokine signals. Another study has implicated LRG-21 in negative T-cell selection (38). The role(s) that LRG-21 plays during drug-induced liver toxicity is unknown. However, one could hypothesize that LRG-21 may play dual roles in the liver: It may be involved in (a) the activation of resident macrophages (Kupffer cells) necessary for the development of APAP-induced liver damage (14, 15) and (b) the modulation of signaling pathways that, in turn, prevent an immune mediated response against drug-protein adducts by inducing apoptosis of drug-specific T cells. These possibilities certainly warrant further study. (5) Suppressor of cytokine signaling (SOCS) proteins are a family of factors that regulate cytokine signal transduction (39). Their expression can be up-regulated in mouse liver with certain stimuli (40) including events associated with APAP toxicity (Table 2; Fig. 1). Recent studies have linked SOCS expression with the down-modulation of macrophage activity (41), suggesting that SOCS induction may be part of an adaptive response limiting the role of resident macrophages (14, 15) in APAP-induced liver toxicity. The fact that SOCS induction may be mediated by IFN-β signaling (41), whose expression also increased following APAP treatment (Table 2), supports this hypothesis. Moreover, IFN-β has been implicated in promoting cell survival via an NFκB-dependent mechanism (42) suggesting, therefore, that IFN-β might act to minimize liver injury through at least two divergent mechanisms. (6) Toxicant-induced tissue injury triggers a cascade of events leading to fibrin deposition that can be beneficial in wound repair but also deleterious to tissue function (43). Whereas plasminogen activators (PA) can prevent fibrin deposition during pathological states (44), plasminogen activator inhibitors (PAI) appear to accumulate during allergic inflammatory states blocking fibrinolysis and promoting collagen deposition and tissue remodeling (45, 46). In fact, genetic deletion of PAI-1 has been shown to protect against bleomycin-induced pulmonary fibrosis indicating its critical involvement in certain forms of tissue injury (43). The dramatic induction of PAI-1 with APAP toxicity (Table 2; Fig. 1) suggests that it may also be involved in

TABLE 3

ESTs without Known Sequence Homology but Whose Expression Was Significantly Altered by APAP Treatment[a]

		Decreased		
aa670835	aa184359	aa238331	aa184286	aa034530
aa221481	11238367	aa217594	aa213083	aa190119
aa204094	aa237412	aa250614	aa105029	aa265415
aa204094	aa237412	aa250614	aa105029	aa265415
aa250708	aa109790	aa183627	aa116735	w40847
aa242579	aa203833	aa250191	aa268163	w30230
		Increased[b]		
aa571570	aa266033	aa409826	aa268619	aa137620
m11686	aa253688	aa388848	aa638759	aa268619
aa688938	aa538285	C81548	aa415488	aa638759
aa002653	aa212550	aa408677	aa571638	aa415488
aa271003	aa104073	aa175784	aa408634	aa571638
C77421	aa198316	AA673251	aa637306	aa408634
aa710439	r75152	aa422356	aa710880	aa637306
aa415488	AA265569	aa185060	aa389999	aa710880
aa175295	aa538477	aa462486	aa614971	aa389999
aa511120	aa184871	aa137620	aa462486	aa614971[c]
aa545236	AA673251			

[a] See Materials and Methods section and Table 1 for exclusion criteria.

[b] Shaded areas indicate that expresses was only detectable in the livers of APAP-treated mice and not in those of saline-treated mice suggesting its induction by APAP treatment.

[c] Boxed area highlights one EST found to have significant homology with a recently identified "molecule possessing anykrin-repeats induced by lipopolysaccharide" (MAIL) factor that potentiates interleukin-6 production (47).

fibrotic events accompanying liver toxicity with a potential role in wound healing and/or fibrosis. Whether the balance between PA and PAI activation is a determinant of the manifestation and extent of drug-induced liver injury warrants further study.

Aside from these and other genes listed in Table 2, expression profiling also unveiled a number of ESTs without known similarities to identified genes that were altered during APAP-induced liver injury (Table 3). Because the microarrays have been designed, in part, to help identify uncharacterized genes, these findings provide the basis for further investigation. Regular searches of GenBank submissions recently demonstrated that one such EST induced by APAP treatment (aa614971) shows significant homology with a recently identified "molecule possessing anykrin-repeats induced by lipopolysaccharide" (MAIL) factor that potentiates interleukin-6 production (47). Although it is unknown what role this factor plays in drug-induced liver injury, it is known that IL-6 plays an important role in the acute phase response, liver regeneration, T- and B-cell differentiation and development (48), as well as protection against carbon tetrachloride-induced liver injury (49). Other ESTs in Table 3 might also turn out to possess activities as yet unexplored in the context of drug-induced liver injury. Availability of the sequenced mouse genome in the very near future should help to address this possibility.

In summary, our expression profiling of APAP hepatotoxicity suggests a wide array of factors with potential importance in drug-induced liver injury. A number of these factors represent novel observations deserving further evaluation. Determination of the expression profile of these and other factors within select cell populations of the liver (i.e., Kupffer cells, endothelial cells, and stellate cells (50)) should help to further delineate the molecular and cellular events involved in drug-induced liver injury. Continued work using these and other complementary approaches, including rapidly evolving proteomics technologies (19, 23), should allow the development of a database of genetic and non-genetic alterations induced during episodes of liver injury that will further our goal of being able to mechanistically understand, and ultimately, predict drug-induced liver toxicity in humans.

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REFERENCES

1. Kaplowitz, N. (2001) *Hepatology* **33,** 308–310.
2. Ray, S. D., and Jena, N. (2000) *Arch. Toxicol.* **73,** 594–606.
3. Gibson, J. D., Pumford, N. R., Samokyszyn, V. M., and Hinson, J. A. (1996) *Chem. Res. Toxicol.* **9,** 580–585.
4. Brent, J. A., and Rumack, B. H. (1993) *J. Toxicol. Clin. Toxicol.* **31,** 173–196.
5. Adamson, G. M., and Harman, A. W. (1993) *Biochem. Pharmacol.* **45,** 2289–2294.
6. Liu, J., Liu, Y., Hartley, D., Klaassen, C. D., Shehin-Johnson, S. E., Lucas, A., and Cohen, S. D. (1999) *J. Pharmacol. Exp. Ther.* **289,** 580–586.
7. Moore, M., Thor, H., Moore, G., Nelson, S., Moldeus, P., and Orrenius, S. (1985) *J. Biol. Chem.* **260,** 13035–13040.
8. Salas, V. M., and Corcoran, G. B. (1997) *Hepatology* **25,** 1432–1438.
9. Burcham, P. C., and Harman, A. W. (1991) *J. Biol. Chem.* **266,** 5049–5054.
10. Blazka, M. E., Germolec, D. R., Simeonova, P., Bruccoleri, A., Pennypacker, K. R., and Luster, M. I. (1995) *J. Inflamm.* **47,** 138–150.
11. Blazka, M. E., Bruccoleri, A., Simeonova, P. P., Germolec, D. R., Pennypacker, K. R., and Luster, M. I. (1996) *Res. Commun. Mol. Pathol. Pharmacol.* **92,** 259–273.
12. Boulares, A. H., Giardina, C., Inan, M. S., Khairallah, E. A., and Cohen, S. D. (2000) *Toxicol. Sci.* **55,** 370–375.
13. Blazka, M. E., Wilmer, J. L., Holladay, S. D., Wilson, R. E., and Luster, M. I. (1995) *Toxicol. Appl. Pharmacol.* **133,** 43–52.
14. Laskin, D. L., Pilaro, A. M., and Ji, S. (1986) *Toxicol. Appl. Pharmacol.* **86,** 216–226.

15. Laskin, D. L., Gardner, C. R., Price, V. F., and Jollow, D. J. (1995) *Hepatology* **21,** 1045–1050.
16. Salminen, W. F., Jr., Voellmy, R., and Roberts, S. M. (1997) *Toxicol. Appl. Pharmacol.* **147,** 247–258.
17. Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E. L. (1996) *Nat. Biotechnol.* **14,** 1675–1680.
18. Wodicka, L., Dong, H., Mittmann, M., Ho, M. H., and Lockhart, D. J. (1997) *Nat. Biotechnol.* **15,** 1359–1367.
19. Aicher, L., Wahl, D., Arce, A., Grenet, O., and Steiner, S. (1998) *Electrophoresis* **19,** 1998–2003.
20. Coller, H. A., Grandori, C., Tamayo, P., Colbert, T., Lander, E. S., Eisenman, R. N., and Golub, T. R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3260–3265.
21. Kaminski, N., Allard, J. D., Pittet, J. F., Zuo, F., Griffiths, M. J., Morris, D., Huang, X., Sheppard, D., and Heller, R. A. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 1778–1783.
22. Puga, A., Maier, A., and Medvedovic, M. (2000) *Biochem. Pharmacol.* **60,** 1129–1142.
23. Fountoulakis, M., Berndt, P., Boelsterli, U. A., Crameri, F., Winter, M., Albertini, S., and Suter, L. (2000) *Electrophoresis* **21,** 2148–2161.
24. Blazka, M. E., Bruccoleri, A., Simeonova, P. P., Germolec, D. R., Pennypacker, K. R., and Luster, M. I. (1996) *Res. Commun. Mol. Pathol. Pharmacol.* **92,** 259–273.
25. Kitteringham, N. R., Powell, H., Clement, Y. N., Dodd, C. C., Tettey, J. N., Pirmohamed, M., Smith, D. A., McLellan, L. I., and Kevin, P. B. (2000) *Hepatology* **32,** 321–333.
26. Fontanier-Razzaq, N. C., Hay, S. M., and Rees, W. D. (1999) *Mol. Reprod. Dev.* **54,** 326–332.
27. Fabbretti, E., Edomi, P., Brancolini, C., and Schneider, C. (1995) *Genes Dev.* **9,** 1846–1856.
28. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) *Genes Dev.* **12,** 982–995.
29. Nakaso, K., Kitayama, M., Fukuda, H., Kimura, K., Yanagawa, T., Ishii, T., Nakashima, K., and Yamada, K. (2000) *Neurosci. Lett.* **282,** 57–60.
30. Ishii, T., Yanagawa, T., Kawane, T., Yuki, K., Seita, J., Yoshida, H., and Bannai, S. (1996) *Biochem. Biophys. Res. Commun.* **226,** 456–460.
31. Okazaki, M., Ito, S., Kawakita, K., Takeshita, S., Kawai, S., Makishima, F., Oda, H., and Kakinuma, A. (1999) *Genomics* **60,** 87–95.
32. Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S., and Yamamoto, M. (2000) *J. Biol. Chem.* **275,** 16023–16029.
33. Devergne, O., Hummel, M., Koeppen, H., Le Beau, M. M., Nathanson, E. C., Kieff, E., and Birkenbach, M. (1996) *J. Virol.* **70,** 1143–1153.
34. Jolly, C., and Morimoto, R. I. (2000) *J. Natl. Cancer Inst.* **92,** 1564–1572.
35. Chandawarkar, R. Y., Wagh, M. S., and Srivastava, P. K. (1999) *J. Exp. Med.* **189,** 1437–1442.
36. Chen, W., Syldath, U., Bellmann, K., Burkart, V., and Kolb, H. (1999) *J. Immunol.* **162,** 3212–3219.
37. Drysdale, B. E., Howard, D. L., and Johnson, R. J. (1996) *Mol. Immunol.* **33,** 989–998.
38. Lerner, A., Clayton, L. K., Mizoguchi, E., Ghendler, Y., van Ewijk, W., Koyasu, S., Bhan, A. K., and Reinherz, E. L. (1996) *EMBO J.* **15,** 5876–5887.
39. Chen, X. P., Losman, J. A., and Rothman, P. (2000) *Immunity* **13,** 287–290.
40. Adams, T. E., Hansen, J. A., Starr, R., Nicola, N. A., Hilton, D. J., and Billestrup, N. (1998) *J. Biol. Chem.* **273,** 1285–1287.
41. Crespo, A., Filla, M. B., Russell, S. W., and Murphy, W. J. (2000) *Biochem. J.* **349,** 99–104.
42. Yang, C. H., Murti, A., Pfeffer, S. R., Basu, L., Kim, J. G., and Pfeffer, L. M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 13631–13636.
43. Hattori, N., Degen, J. L., Sisson, T. H., Liu, H., Moore, B. B., Pandrangi, R. G., Simon, R. H., and Drew, A. F. (2000) *J. Clin. Invest.* **106,** 1341–1350.
44. Cho, S. H. Tam, S. W., Demissie-Sanders, S., Filler, S. A., and Oh, C. K. (2000) *J. Immunol.* **165,** 3154–3161.
45. Zhang, L. P., Takahara, T., Yata, Y., Furui, K., Jin, B., Kawada, N., and Watanabe, A. (1999) *J. Hepatol.* **31,** 703–711.
46. Seki, T., Healy, A. M., Fletcher, D. S., Noguchi, T., and Gelehrter, T. D. (1999) *Am. J. Physiol.* **277,** G801–G809.
47. Kitamura, H., Kanehira, K., Okita, K., Morimatsu, M., and Saito, M. (2000) *FEBS Lett.* **485,** 53–56.
48. Streetz, K. L., Luedde, T., Manns, M. P., and Trautwein, C. (2000) *Gut* **47,** 309–312.
49. Kovalovich, K., DeAngelis, R. A., Li, W., Furth, E. E., Ciliberto, G., and Taub, R. (2000) *Hepatology* **31,** 149–159.
50. Kristensen, D. B., Kawada, N., Imamura, K., Miyamoto, Y., Tateno, C., Seki, S., Kuroki, T., and Yoshizato, K. (2000) *Hepatology* **32,** 268–277.