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Original Articles

Screening for Drug-Induced Hepatotoxicity in Primary Mouse Hepatocytes Using Acetaminophen, Amiodarone, and Cyclosporin A as Model Compounds: An Omics-Guided Approach

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Abstract

Drug-induced hepatotoxicity is a leading cause of attrition for candidate pharmaceuticals in development. New preclinical screening methods are crucial to predict drug toxicity prior to human studies. Of all *in vitro* hepatotoxicity models, primary human hepatocytes are considered as 'the gold standard.' However, their use is hindered by limited availability and inter-individual variation. These barriers may be overcome by using primary mouse hepatocytes. We used differential in gel electrophoresis (DIGE) to study large-scale protein expression of primary mouse hepatocytes. These hepatocytes were exposed to three well-defined hepatotoxicants: acetaminophen, amiodarone, and cyclosporin A. Each hepatotoxicant induces a different hepatotoxic phenotype. Based on the DIGE results, the mRNA expression levels of deregulated proteins from cyclosporin A-treated cells were also analyzed. We were able to distinguish cyclosporin A from controls, as well as acetaminophen and amiodarone-treated samples. Cyclosporin A induced endoplasmic reticulum (ER) stress and altered the ER-Golgi transport. Moreover, liver carboxylesterase and bile salt sulfotransferase were differentially expressed. These proteins were associated with a protective adaptive response against cyclosporin A-induced cholestasis. The results of this study are comparable with effects in HepG2 cells. Therefore, we suggest both models can be used to analyze the cholestatic properties of cyclosporin A. Furthermore, this study showed a conserved response between primary mouse hepatocytes and HepG2 cells. These findings collectively lend support for use of omics strategies in preclinical toxicology, and might inform future efforts to better link preclinical and clinical research in rational drug development.

Introduction

The liver is responsible for detoxification and elimination of potentially harmful substances. It is an important target organ for xenobiotic compounds. For this reason, hepatotoxicity is the most prominent adverse drug reaction leading to the failure of candidate drugs in preclinical or clinical trials. New screening methods, which can detect drug-induced liver injury at an early stage of the drug development, represents an important step towards rational drug development.

In xenobiotic metabolism and toxicity studies, primary hepatocytes are considered as 'the gold standard' of *in vitro* models (Hewitt et al., 2007). The isolation of primary hepatocytes was introduced by Seglen (1976). He developed the two-step collagenase perfusion protocol to obtain viable rat hepatocytes. Primary rat and, to a lesser extent, human hepatocytes, are currently established *in vitro* systems. Previously it was shown that primary human hepatocytes have a similar expression pattern of the biotransformation genes as human liver tissue (Jennen et al., 2010). However, the use of primary human hepatocytes is hindered by the scarcity of suitable liver samples and by considerable inter-individual variation due to genetic, environmental, and age differences of the donors (Donato et al., 2008). Furthermore, the longer the culturing, the human as well as the alternative primary rat hepatocytes show a relatively rapid decline of their cytochrome P450 enzyme activities (Boess et al., 2003). Based on their gene expression and cytochrome P450 enzyme activity, primary mouse hepatocytes maintain their metabolic

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competence better than to rat hepatocytes (Mathijs et al., 2009b). Moreover, transgenic mouse models are widely available, which may provide primary hepatocytes suitable for dedicated mechanistic investigations of liver toxicity. For instance, hepatocytes from the DNA repair-deficient $Xpa^{-/-}$ $p53^{+/-}$ mouse have been considered as a model for carcinogenicity screening (van Kesteren et al., 2011). As such, primary mouse hepatocytes seem a promising model for hepatic toxicity studies.

Beside primary hepatocytes, hepatic carcinoma cell lines as HepG2 cells are established models for toxicity studies. It has been shown that HepG2 cells are able to metabolize xenobiotic compounds leading to toxic effects, including genotoxicity, oxidative stress, and mitochondrial dysfunction (Knasmuller et al., 2004, O'Brien et al., 2006, Schoonen et al., 2005). However, it is undeniable that these cells have lost some liver specific functions due to immortalization, in particular the phase I drug metabolizing enzymes (Boess et al., 2003, Wilkening and Bader, 2003).

The 'omics' technologies provide powerful tools for expression profiling of biological events, and have the potential to improve current toxicity tests (Aardema and MacGregor, 2002). The conventional toxicity tests mostly rely on the examination of clinical, hematological, and histopathological parameters. It can take weeks, months, or even years before these traditional toxicological endpoints occur. Specific changes in protein and mRNA expression could occur within a few hours or days after exposure to chemical compounds.

Within the 'omics' field, the whole genome gene expression analysis is still the driving technology. But it is acknowledged that the relative gene expression levels often only moderately correlate with the relative abundance of its protein product. This moderate correlation is due to the turnover differences of proteins and mRNA (Greenbaum et al., 2003). Moreover, post-translational modifications and protein interactions are not detected by transcriptomics. This emphasizes the need for proteomics, for example, with differential in gel electrophoresis (DIGE) proteins are separated based on their pI and molecular weight, so different protein isoforms can be visualized.

Previously we studied the proteome of HepG2 cells after incubation with three well-defined hepatotoxicants: namely acetaminophen, amiodarone, and cyclosporin A. They each represent a different class of hepatotoxicity (Van Summeren et al., 2011). In this study we investigated whether hepatotoxicants, inducing different toxicological phenotypes, produce distinct differences in the protein expression of HepG2 cells. Compared with the other compounds, cyclosporin A induced the most prominent effect on the proteome of the HepG2 cells. Because the use of primary hepatocytes is preferred in toxicological studies, we also wanted to analyze the proteome of primary mouse hepatocytes for its ability to discriminate between different types of drug-induced hepatotoxicity, and the level of conservation of response in comparison with a human *in vitro* model. For that reason, we analyzed the protein expression in primary mouse hepatocytes after exposure to acetaminophen, amiodarone, and cyclosporin A, and compared it with the protein expression in HepG2, obtained from our previous study (Van Summeren et al., 2011). To investigate the correlation between the protein and mRNA expression, we compared the differentially expressed proteins induced by cyclosporin A, with their corresponding mRNA expression.

Materials and Methods

Chemicals

Dulbecco's Modified Eagle's medium, fetal calf serum, penicillin/streptomycin, Hanks' calcium- and magnesium-free buffer and insulin were obtained from Life Technologies (Breda, The Netherlands). Glucagon, hydrocortisone (water soluble), collagenase type IV, dimethylsulfoxide (DMSO), trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NaCl, $NaHCO_3$, KCl, KH_2PO_4; $MgSO_4$, glucose; $CaCl_2$, acetaminophen, amiodarone, cyclosporin A (BioChemika), and N,N-dimethylformamide (anhydrous, 99.8%) were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Collagen Type I Rat Tail was obtained from BD BioSciences (Bedford, MA, USA), the Protein Assay Kit and nonfat dry milk powder (NFDM) was from Bio-Rad (Veenendaal, The Netherlands). All chemicals used for DIGE were purchased from GE Healthcare (Diegem, Belgium). The antibody against perilipin2/adipophilin (Plin2) used for Western blotting was purchased from Abcam (Cambridge, UK). The antibodies against β-actin and bile sulfotransferase were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The horseradish peroxidase-conjugated secondary antibodies rabbit anti-mouse and swine anti-rabbit were obtained from DAKO (Enschede, The Netherlands). The chemiluminescent substrate (SuperSignal CL) was purchased from Thermo Fisher Scientific (Etten-Leur, The Netherlands). The Trizol reagent and the RNeasy mini kit were from Qiagen Westburg (Leusden, The Netherlands).

Animals

Permission for animal studies was obtained from the Animal Ethical Committee of the Maastricht University, The Netherlands (approval number: 2008-075). Adult male C57/B6 mice, weighing 20–25 g, were obtained from Charles River GmbH, Sulzfeld, Germany. The animals were housed in macrolon cages with sawdust bedding at 22°C and 50%–60% humidity. The light cycle was 12 h light/12 h dark. Food and tap water were available *ad libitum*.

Isolation and culturing of primary mouse hepatocytes

Hepatocytes were isolated by a two-step collagenase perfusion method according to Seglen (1976), with modifications as described before (Mathijs et al., 2009b).

Cell suspensions with cell viability $\geq 80\%$, determined by trypan blue exclusion, were brought into culture in a collagen-collagen sandwich as described before (Mathijs et al., 2009b). Prior to treatment, the primary mouse hepatocytes were allowed to recover for 40–42 h at 37°C in a humidified chamber with 95%/5% air/CO_2 in serum-free culture medium supplemented with insulin (0.5 U/mL), glucagon (7 ng/mL), hydrocortisone (7.5 l g/mL), and 2% penicillin/streptomycin (5000 U/ml penicillin and 5000 lm/mL streptomycin). Culture medium was refreshed every 24 h. After the recovery period, the culture medium was replaced by the culture medium containing one of the selected compounds or 0.5% DMSO as a vehicle control. For each compound, the IC_{20} concentration was determined by the MTT reduction method (Mosmann, 1983). Based on the IC_{20} values, the hepatocytes were exposed as follows: 1 mM acetaminophen, 1 μM amiodarone, and 10 μM cyclosporin A or 0.5 % DMSO as a vehicle

control for 48 h. For proteome analysis, five independent biological experiments, each with cells from a different animal, were performed. For transcriptomics, three new independent biological experiments were conducted.

Sample preparation

The cells were washed twice with PBS. For the protein extraction of the hepatocytes, the collagen layers were removed to prevent its interference with the proteome analysis. Intact cells were isolated from the collagen layers after 10 min incubation with collagenase buffer. The collagenase buffer contains 1150 CDU/100 mL collagenase (CDU = collagen digestion units) dissolved in a Krebs buffer (118 mM NaCl; 25 mM $NaHCO_3$; 4.8 mM KCl; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 11 mM glucose; 1.5 mM $CaCl_2$). The suspension of detached cells was washed and further diluted till 50 mL with PBS, and centrifuged for 5 min at 65 *g*. To wash the cells thoroughly, the washing step was repeated. Afterwards the supernatant was removed and the cell pellet was dissolved in a DIGE labeling buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 30 mM Tris-HCl. This mixture was subjected to three cycles of freeze thawing with liquid nitrogen, vortexed thoroughly and centrifuged at 20,000 *g* for 30 min at 10°C. Supernatant was collected, aliquoted, and stored at −80°C until further analysis. Protein concentrations were determined with the Protein Assay Kit from Bio-Rad (Veenendaal, The Netherlands).

The total RNA from hepatocytes treated with cyclosporin A was isolated, using Trizol reagent with the RNeasy kit, according to the manufacturer's protocol. Total cellular RNA levels were measured with a spectrophotometer and the quality of each RNA preparation was determined with a bioanalyzer (Agilent Technologies, The Netherlands). Extracted RNA was stored at −80°C.

Differential in gel electrophoresis

The protein labeling and the DIGE were performed as described before (Van Summeren et al., 2011). A one-way ANOVA test ($p \leq 0.05$) was used to select the significant differential spots between the experimental groups. The EDA module of the DeCyder software was also used to perform a hierarchical clustering analysis and a Principle Component Analysis (PCA). The differentially expressed proteins ($p \leq 0.05$) were excised and identified by a MALDI-TOF/TOF mass spectrometer (Bouwman et al., 2009). Protein spots that could not be identified via MALDI-TOF MS were further analyzed by nano liquid chromatography tandem mass spectrometry (LC-MSMS) on an LCQ Classic (ThermoFinnigan) as described (Dumont et al., 2004).

Western blot analysis

Samples with equal amount of protein (30 μg/lane) were separated by SDS-PAGE on 4%–12% Bis-Tris Criterion gels (Bio-Rad, Hercules, CA), at 150 V. and transferred to a 0.45 mm nitrocellulose membrane for 90 min at 100 V. After Ponceau S staining and destaining, membranes were blocked in 5% NFDM in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h. Thereafter, the blots were incubated with the primary antibodies against adipophilin (1:500 dilution), in 5% NFDM-TBST overnight at 4°C on a shaker. The blot was washed three times for 10 min in TBST and then incubated for 1 h with a 1:10,000 dilution of the horseradish peroxidase-conjugated secondary antibody in 5% NFDM-TBST. The blots were washed three times for 10 min in TBST. To detect bile salt sulfotransferase (Sult2A1), the same procedure was repeated, except for blocking (5% BSA) and the dilution of the first antibody (1:200). A CCD camera (XRS-system, Bio-Rad) was used to detect immunoreactive bands using chemiluminescent substrate (SuperSignal CL). The quantification was performed with the program Quantity One version 4.6.5 (Bio-Rad). β-Actin was used as reference for the amount of protein loaded.

Microarray analysis

The targets were prepared according to the Affymetrix protocol. Data analysis was performed as described earlier (Mathijs et al., 2009a). Only the genes of the differentially expressed proteins were selected and retrieved from the transcriptome data. Fold change calculations and student *t*-tests were performed in Microsoft Excel. Differentially expressed genes with a p value ≤ 0.05 and a fold change ≥ 1.5 were considered as significant.

Results

Effect of the hepatotoxicants on the proteome of primary mouse hepatocytes

The cellular proteins of the treated primary mouse hepatocytes were analyzed using DIGE. In total, 1866 spots could be matched with all the images. With a one-way ANOVA analysis ($p \leq 0.05$), 53 spots were detected as significantly differential. Based on the Tukey's multiple comparison test, significantly differential protein expression was only observed with cyclosporin A, but not with acetaminophen and amiodarone. The experimental groups (control, acetaminophen, amiodarone, and cyclosporin A) were clustered based on the log standard abundance of the 53 differential spots with a hierarchical clustering algorithm. As shown in Figure 1, the spot maps of cyclosporin A are distinguished mostly from other spot maps. Differences were also found between the control, acetaminophen, and amiodarone, although they were rather small, causing them to cluster together. A PCA was performed on the 53 differential spots (Fig. 2). The cyclosporin A-treated hepatocytes were discriminated by PC1 accounting for a variance of 44.3%.

Protein identification from the differential spots

The differential spots were included in a pick list and excised from a preparative gel. Protein identification was performed by in-gel digestion followed by MALDI-TOF/TOF tandem MS and/or LC MS/MS (Supplemental Data s1, Supporting Information). Out of the 53 spots, the proteins of 43 spots were identified (Table 1). Figure 3 shows the 2-DE map made from the master gel with the 43 identified differential spots indicated with a number that corresponds to the numbers presented in Table 1.

For spot numbers 1, 11, 14, and 17, multiple proteins for one spot were identified with LC MS/MS, both protein identifications delivering the same number of peptides. Consequently, for these spots it is not possible to conclude which protein is responsible for the significant change of the fold change.

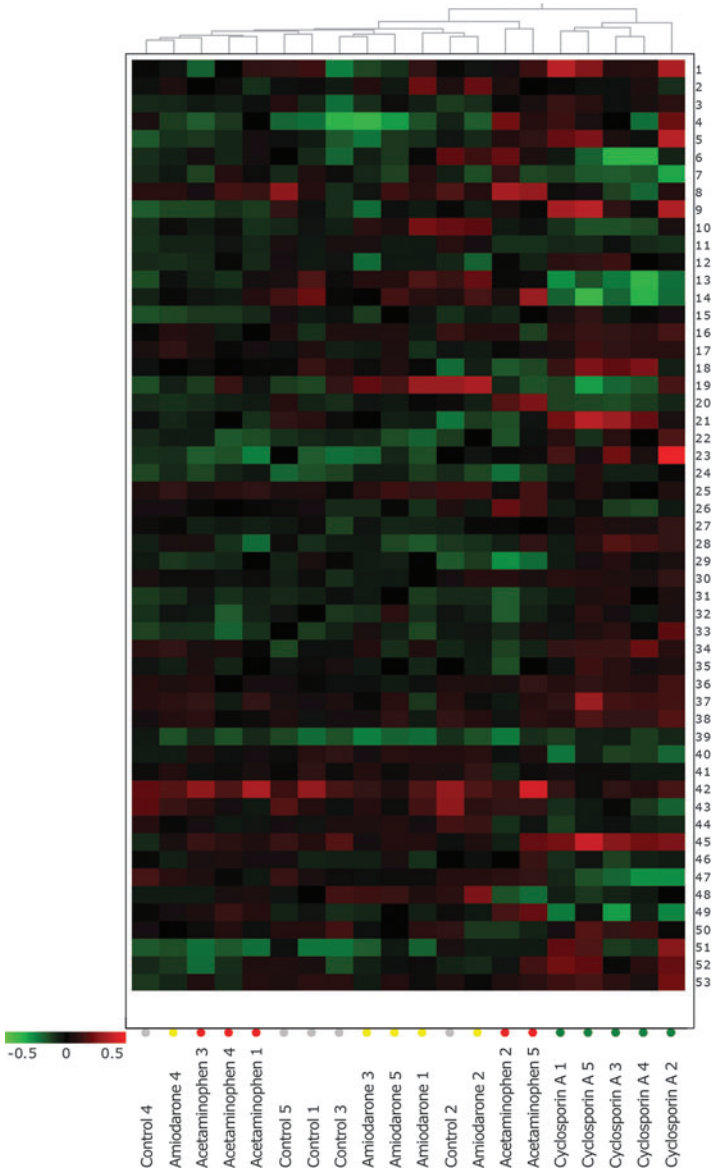

FIG. 1. Hierarchical cluster analysis of the experimental groups (control, acetaminophen, amiodarone, and cyclosporin A). The clustering is based on the log standard abundance of the significant differential spots ($p \leq 0.05$) with a hierarchical clustering algorithm in the EDA module of the Decyder software.

Nine spots appeared isoforms from four proteins due to post-translational modifications or processing of the protein. The functional properties of the identified proteins were obtained by the Panther classification system (http://www.pantherdb.org). The majority of the differential proteins are involved in transport, metabolic and cellular processes (Fig. 4).

To confirm our findings obtained with the DIGE analysis, Western blot analysis was performed on two significantly changed proteins, Plin2 and Sult2A1. Because of their role in cholesterol metabolism, these proteins are probably important for the mechanism behind cyclosporin A-induced cholestasis.

The DIGE analysis showed a decreased expression of Plin2 after cyclosporin A treatment. This was confirmed by Western blotting, which showed a significantly decreased expression of this protein after cyclosporin A treatment with a p value of 0.037 (Fig. 5A). An insignificant decrease of this protein was found after treatment with the other hepatotoxicants. The DIGE analysis revealed an increased expression of Sult2A1 upon cyclosporin A treatment. The Western blot showed a significantly increased expression for all three drugs with p values of 0.0016, 0.030, and 0.043 for acetaminophen, amiodarone, and cyclosporin A, respectively (Fig. 5B)

Correlation of the differentially expressed proteins with RNA expression

The mRNA levels of the differentially expressed proteins from cyclosporin A-treated cells were retrieved from the micro-array data. We focused on the cyclosporin A-treated cells, since all differentially expressed proteins were assigned to cyclosporin A. For the following significantly upregulated proteins, a corresponding upregulation of their mRNA was found: protein disulfide isomerase, protein disulfide

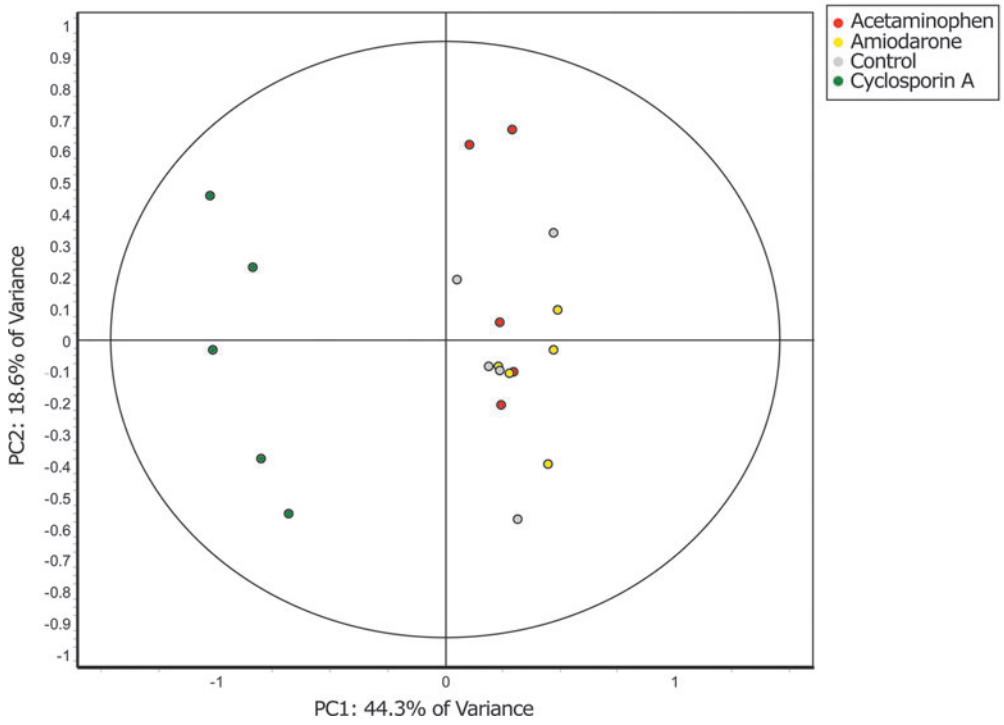

FIG. 2. PCA analysis of the experimental groups (control, acetaminophen, amiodarone, and cyclosporin A), based on the significant differential spots (One-Way Anova $p \leq 0.05$).

isomerase A6, protein disulfide isomerase A4, keratin type I cytoskeletal 10, 78 kDa glucose-regulated protein, and mesencephalic astrocyte-derived neurotrophic factor. Liver carboxylesterase 1 (Ces1) showed a significant decrease on the proteome and transcriptome induced by cyclosporin A. Furthermore, cyclosporin A also downregulated protein expression of alanine aminotransferase 2 (Gpt2), however not significant according to the multiple comparison test. On the other hand, Gpt2 was significantly downregulated on transcriptome. These data suggest that cellular changes due to a 48-h cyclosporin A treatment can be the consequence of transcriptional adaptations. However, divergences between protein and RNA expression were also observed. For instance, the protein expression of ornithine carbamoyltransferase was found upregulated with a fold change of 2.12, while the RNA expression of the same protein was found highly downregulated with a fold change of −6.53. Differences in protein and RNA expression point out altered gene expression regulation at the translational or post-translational level. No clear indication for post-translational regulation was obtained, since the detected isoforms from Plin2 and 78 kDa glucose-regulated protein changed all in the same direction.

Discussion

In this study, the proteome of primary mouse hepatocytes was assessed for its ability to discriminate between different phenotypes of drug-induced hepatotoxicity, and the level of conservation in response when compared to a human *in vitro* model was determined.

Three well-characterized hepatotoxicants: acetaminophen, amiodarone, and cyclosporin A were investigated. Each compound induces a different hepatotoxic phenotype, making it possible to analyze the differences in the pathways which are specific for different classes of hepatotoxicants. Acetaminophen is a widely known and used analgesic, it is safe at a therapeutic dose, but causes severe damage to the liver at higher doses and therefore it is applied as a reference compound for necrosis (Murray et al., 2008). Cycloporin A is a immunosuppressive drug that has been shown to induce cholestasis (Rotolo et al., 1986). The anti-arrhythmic drug, amiodarone, induces steatosis as side effect (Fromenty et al., 1990). The primary mouse hepatocytes were exposed for 48 h to the test compounds and afterwards changes in protein expression were studied. Significantly differentially expressed proteins were only found for cyclosporin A-treated cells. The differentially expressed spots were used in a hierarchical clustering analysis where cyclosporin A could be distinguished from acetaminophen and amiodarone. This result is in agreement with our previous study where HepG2 cells were exposed to the same compounds. However, primary mouse hepatocytes show a considerably fewer amount of differentially expressed proteins compared with HepG2 cells, namely 53 spots versus 254 spots (Van Summeren et al., 2011). This result was unexpected, since primary hepatocytes have a higher metabolic activity and liver specificity than HepG2 cells (Gerets et al., 2012). One explanation may be the larger biological variation between the primary mouse hepatocytes compared to HepG2 cells, leading to the detection of a smaller number of significantly changed spots.

Furthermore, just like HepG2, the proteome of the primary mouse hepatocytes was not able to make an adequate differentiation between amiodarone, acetaminophen, and the control samples. For each *in vitro* model, the dosages of the compounds were based on the IC_{20} concentrations determined with a MTT assay. We used individual IC_{20}

Table 1. Protein Identification of Differentially Expressed Proteins in Primary Mouse Hepatocytes after Exposure to Acetaminophen, Amiodarone, or Cyclosporin A and the mRNA Expression of the Differentially Expressed Proteins after Exposure to cyclosporin A

			Protein expression						*mRNA expression*	
			p *value (one-way anova)*[a]	*Fold change*[b]					p *value (T test)*	*Fold change CsA/C*
No.	*uniprot*	*Protein description*		*CsA/C*	*Ac/C*	*Am/C*	*HepG2*	*Gene*		
Metabolic process										
1	P17182	Alpha-enolase	0.000117	−2.25*	1	−1	yes	Eno1	#N/A	N/A
1	A2RSX9	Arfaptin-1 protein	0.00012	−2.25*	1	−1		Arfip1	0.790	−1.02
2	P11725	Ornithine carbamoyltransferase, mitochondrial	0.00385	2.12*	−1.02	−1.03		Otc	0.0336	−6.53‡
5	P09103	Protein disulfide-isomerase	0.01132	1.89*	−1.08	−1.16		P4hb	0.00208	1.64‡
11	Q9D1T5	Proline-rich protein 15	0.00109	1.63*	1.07	−1.04		Prr15	0.711	−1.03
11	Q80ZP8	Mesencephalic astrocyte-derived neurotrophic factor	0.00109	1.63*	1.07	−1.04		Manf	0.00618	3.55‡
13	P40936	Indolethylamine N-methyltransferase	0.019	1.54*	1.06	−1.03		Inmt	0.182	−1.26
14	P27773	Protein disulfide-isomerase A3	0.022	−1.46*	1.05	−1.07	yes	Pdia3	0.0730	1.46
15	P16858	Glyceraldehyde-3-phosphate dehydrogenase isoform 1	0.0426	−1.46	1.18	1.02		Gapdh	#N/A	N/A
16	P52843	Bile salt sulfotransferase	0.0229	1.45	1.06	−1.05		Sult2a1	#N/A	N/A
17	Q60854	Serpin B6	0.0288	1.43	−1.16	−1.01		SERPINB6	0.0311	1.68‡
18	Q8BGT5	Alanine aminotransferase 2	0.0418	−1.41	1.19	−1.11		Gpt2	0.000636	−1.89‡
19	P08003	Protein disulfide-isomerase A4	0.00991	1.4*	1.17	1.05	yes	Pdia4	0.0248	1.84‡
25	P60843	Eukaryotic initiation factor 4A-I	0.045	−1.26	1.24	−1.04	yes	Eif4a1	#N/A	N/A
26	Q922R8	Protein disulfide-isomerase A6	0.046	1.25	1.01	−1.09	yes	Pdia6	0.0118	2.30‡
32	P70195	Proteasome subunit beta type-7	0.0302	1.22*	1.09	1.04		Psmb7	0.0598	1.12
33	P54775	26S protease regulatory subunit 6B	0.0427	1.21	−1.06	−1.03		Psmc4	0.299	−1.09
34	P16460	Argininosuccinate synthase	0.025	1.21	−1.1	1.02		ass1	#N/A	N/A
42	P00375	Dihydrofolate reductase	0.0386	−1.1	−1.1	1.06		Dhfr	0.0430	−1.68‡
43	Q8C196	Carbamoyl-phosphate synthase	0.0471	−1.02	−1.02	1.3		Cps1	#N/A	N/A
Cellular response										
20	O70400	PDZ and LIM domain protein 1	0.0195	1.39	−1.04	1		Pdlim1	0.0479	1.54‡
21	P60710	Actin, cytoplasmic 1	0.04	−1.37	−1.19	1.48	yes	Actb	0.0276	−1.12
22	P02535	Keratin, type I cytoskeletal 10	0.013	1.35*	1.07	1.14	yes	Krt10	0.0313	1.61‡
23	P11679	Keratin, type II cytoskeletal 8	0.00909	−1.28	−1.1	1.31	yes	Krt8	0.101	−1.34
32	Q99P30	Isoform 1 of peroxisomal coenzyme A diphosphatase NUDT7	0.0302	1.22*	1.09	1.04		Nudt7	0.120	−1.38
37	P67778	Prohibitin	0.0449	1.16	−1.21	−1.06		phb	0.0319	−1.28

(*continued*)

Table 1. (Continued)

			Protein expression						mRNA expression	
				Fold change[b]						
No.	*uniprot*	*Protein description*	p *value (one-way anova)*[a]	*CsA/C*	*Ac/C*	*Am/C*	*HepG2*	*Gene*	p *value (T test)*	*Fold change CsA/C*
38	D3Z5W7	Glutathione S-transferase, theta 1	0.013	1.16	1.04	−1.06		Gstt1	0.0219	−3.28‡
36	Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial	0.0351	−1.18	1.18	−1.11		Etfa	0.0135	−1.42
40	P56395	Cytochrome b5	0.00698	−1.13	1.16	−1.04		Cyb5a	0.0367	−1.34
41	P35700	Peroxiredoxin-1	0.0373	1.11	1.01	−1.17		Prdx1	0.600	1.03
Transport										
1	P43883	Perilipin2/adipophilin	0.00012	−2.25*	1	−1		Plin2	0.177	−1.16
3	P43883	Perilipin2/adipophilin	6.19E-05	−2.01*	−1.17	1.14		Plin2	0.177	−1.16
4	P24369	Peptidyl-prolyl cis-trans isomerase B	0.000011	−1.9*	−1.08	−1.12		Ppib	0.0178	1.19
8	P24369	Peptidyl-prolyl cis-trans isomerase B	0.00298	−1.68*	−1.21	−1.14		Ppib	0.0178	1.19
6	P07724	Serum albumin precursor	0.00147	1.86*	1.22	−1.11		Alb	0.0714	−1.07
12	P07724	Serum albumin	0.0363	1.57	1.46	−1.21	yes	Alb	0.0714	−1.07
9	Q921I1	Serotransferrin precursor	0.0267	1.65*	1.01	−1.11	yes	Tf	0.0765	−1.17
24	Q9D6F9	Tubulin beta-4 chain	0.00229	1.28	−1.01	−1.18		Tubb4	0.908	1.03
28	Q60930	Voltage-dependent anion-selective channel protein 2	0.00886	1.24	1.02	1.01		Vdac2	0.498	1.04
29	Q99J08	SEC14-like protein 2	0.031	1.23	1.05	−1.01		Sec14l2	0.0190	−2.30‡
Response to stimulus										
7	P20029	78 kDa glucose-regulated protein	0.000763	1.69*	−1.18	−1.17	yes	Hspa5	0.00323	2.11‡
10	P20029	78 kDa glucose-regulated protein	0.045	1.64*	−1.06	−1.01	yes	Hspa5	0.0032	2.11‡
17	P20029	78 kDa glucose-regulated protein	0.0288	1.43	−1.16	−1.01	yes	Hspa5	0.00323	2.11‡
14	Q8VCC2	Liver carboxylesterase 1	0.022	−1.46*	1.05	−1.07	yes	Ces1	0.0396	−6.27‡
27	Q99KB8	Hydroxyacylglutathione hydrolase, mitochondrial	0.00864	1.24*	−1.04	1.01		Hagh	0.00437	1.40
30	P30115	Glutathione S-transferase Yc	0.037	1.23	−1.01	1.01		Gsta3	0.0434	−1.85‡
31	Q63836	Selenium-binding protein 2	0.038	−1.22	−1.22	−1.06		Selenbp2	#N/A	N/A
35	P14602	Isoform A of Heat shock protein beta-1	0.043	1.19	−1.12	1.06		Hspb1	#N/A	N/A
39	Q9WVL0	Maleylacetoacetate isomerase	0.0252	1.15	−1.07	−1.1		Gstz1	0.0574	−2.10

[a]*p* value from one way ANOVA statistical test between the four groups with each five biological replicates; [b]The difference in the standardized abundance of the proteins is expressed as the fold change between the control (C) and the treated groups (T). The fold change is calculated by taking the means of standardized volume values for the protein spot in the corresponding groups (Ac, acetaminophen; Am, amiodarone; C, control; CsA, cyclosporin A), values are calculated as T/C and displayed in the range of +1 to +∞ for increases in expression and calculated as −C/T and displayed in the range of −∞ to −1 for decreased expression; *significant fold changes ($P \leq 0.05$) between the control and the treated group, calculated with a multiple comparison test; ‡significant fold changes ($p \leq 0.05$; $|FC| \geq 1.5$) between the control and the cyclosporin A treated group, calculated with a Student *T* test.

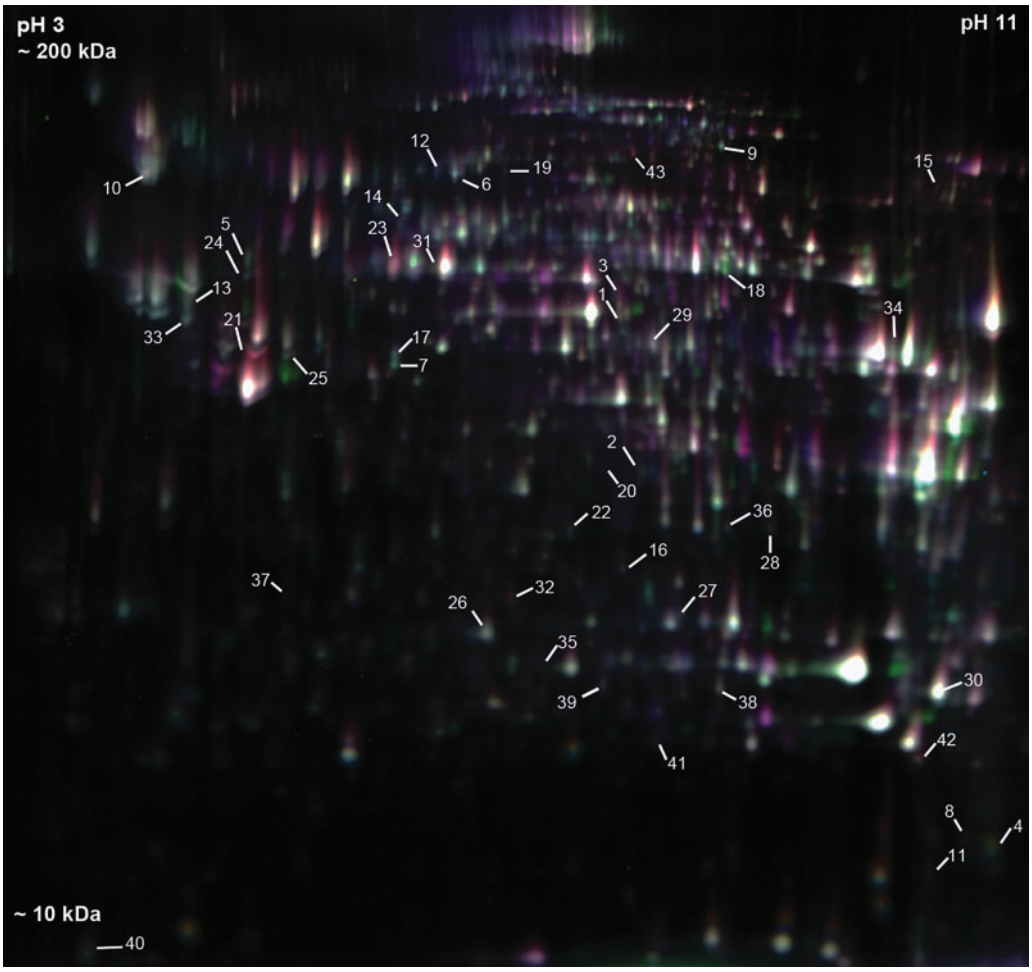

FIG. 3. Proteome map of the differentially expressed proteins (One-Way Anova $p \leq 0.05$). All the identified spots are indicated with a number which corresponds to the numbers used in Table 1.

concentrations because each models can differ in sensitivity to xenobiotic compounds due to their differences in metabolic activity. Probably the IC_{20} of acetaminophen and amiodarone induced relatively small effects *in vitro* that were not detectable with the applied DIGE method. Furthermore, both acetaminophen and amiodarone are indirect toxins, which require biotranformation for toxicity. Acetaminophen is metabolized by the CYP P450 enzymes 2E1, 1A2, and 3A4 to its reactive intermediate N-acetyl *p*-benzoquinoneimine, (NAPQI), which is the main metabolite responsible for the toxicity of acetaminophen (Bessems, and Vermeulen, 2001, Patten et al., 1993). Amiodarone is extensively metabolized in the liver by CYP 3A4 to its toxic mono-N-desethyl and di-N-desethylmetabolites (Zahno et al., 2010). Although primary mouse hepatocytes are a robust model with drug metabolizing capacities, they do show a decline of the CYP 1A2, 3A4, and 2E1 expression with increased cultivation time (Mathijs et al., 2009b). Probably the decreased expression of these drug metabolizing enzymes leads to less toxic metabolites. So only a relatively small effect can be observed.

We observed 12 differentially expressed proteins that overlap between the primary mouse hepatocytes in the present study and the previously analyzed HepG2 cells (Fig. 6). These proteins are: Eno1, Hspa5, Tf, Alb, Ces1, Pdia3, Pdia4, Pdia6, Actb, Krt10, Krt8, and Eif4a1. Moreover, a classification based on the GO-terms of the differentially expressed proteins with the Panther classification system (http://www.pantherdb.org) revealed a similar outcome (Fig. 4). Although different sets of proteins were found differentially expressed, the same pathways in both *in vitro* systems seem to be affected by cyclosporin A. This strongly indicates that the protein expression induced by the hepatotoxicants, cyclosporin A in particular, is conserved in primary mouse hepatocytes when compared to HepG2 cells.

Cyclosporin A is a strong immunosuppressant and induces cholestasis as adverse reaction (Belin et al., 1990). It is known that cyclosporin A inhibits the bile salt export pump (ABCB11), multidrug resistance protein 2 (ABCC2), and P-glycoprotein (ABCB1) in canalicular membrane vesicles. These ATP Binding Cassette transporters (ABC transporters) are responsible for the secretion of bile components into the bile canaliculus (Trauner and Boyer, 2003). Therefore, inhibition of these transport proteins will hamper the bile secretion, which results in cholestasis (Alrefai and Gill, 2007).

Production and secretion of bile acids is the major route for the elimination of excessive cholesterol (Zhao et al., 2005). Consequently, cyclosporin A not only induces the accumulation of bile acids but it also increases the hepatic pool of free cholesterol. Excess of free cholesterol is esterified with long-chain fatty acids by acyl-CoA cholesterol acyltransferase-2

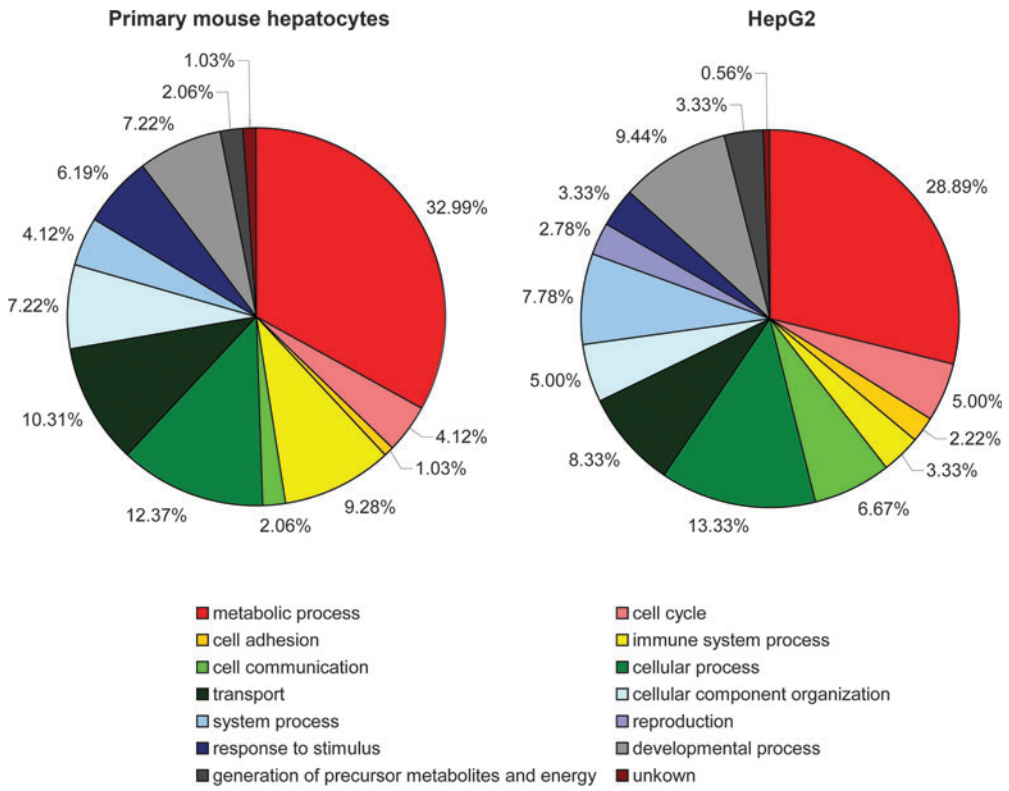

FIG. 4. Classification of the differential expressed proteins in HepG2 and primary mouse hepatocytes after exposure to cyclosporin A with the Panther classification system (http://www.pantherdb.org).

(ACAT2). These cholesteryl esters either establish as a part of the neutral lipid core of very low-density lipoprotein (VLDL) or are accumulated as cytoplasmic lipid droplets (Zhao et al., 2005).

Previously, it was shown that cyclosporin A induced an increase of hepatic VLDL triglyceride secretion (Wu et al., 1999), and a decrease of high-density lipoprotein (HDL) plasma levels. The secretion of VLDL, containing cholesteryl esters, might be an alternative pathway for the removal of excessive cholesterol. Consistent with an increased triglyceride secretion is our observation that Plin 2, a major protein for storage of triglycerides, was found decreased after cyclosporin A treatment. In adipocytes, it was also shown that a decreased expression of Plin 2 was accompanied with an increased secretion of VLDL (Magnusson et al., 2006).

The cholesteryl esters, stored in intracellular lipid droplets, can be hydrolyzed by Ces1. So they become available for bile acid synthesis (Zhao et al., 2005). Here, cyclosporin A induced downregulation of Ces1 on the proteome and transcriptome of primary mouse hepatocytes. A decreased expression of Ces1 will lower the amount of free cholesterol and subsequently decrease the amount of bile acids in the hepatotocytes, which can be seen as a protective adaptive response against cyclosporin A-induced cholestasis.

The phase II detoxifying enzymes are responsible for another mechanism to lower the amount of intracellular bile acids. By means of a conjugation reaction (e.g., sulfation, acetylation, and glucuronidation), these biotransformation enzymes convert xenobiotics or endogenous products into more easily excretable substances (Jancova et al., 2010).

Our study shows an increased expression of the Sult2A1 as a response to cyclosporin A treatment. This phase II detoxifying enzyme catalyzes the sulfation of steroids and bile acids in the liver to increase their polarity and to enhance renal and fecal excretion (Chen and Segel, 1985). Consequently, sulfation of bile salts is linked to a possible protective mechanism against monohydroxy bile salts (Cowen et al., 1975).

Two forms of glutathione-S-transferase (Gsta3 and GSTT1) were downregulated at the mRNA level, but upregulated at the protein level. The difference in protein expression may indicate protein modifications of these enzymes. One could expect an increased expression of these phase II detoxifying enzymes in response to chemical compounds. However, rats treated with cyclosporin A also showed a decreased mRNA expression of hepatic glutathione-S-transferase and two glutathione producing enzymes (γ-glutamylcysteine synthetase heavy and light chain) (Bramow et al., 2001).

The selenium-binding (Selenbp 2) protein, a major target of reactive acetaminophen metabolites, was also differentially expressed. Fountoulakis et al. (2000) reported a decrease of Selenbp 2 after acetaminophen treatment. In this study we also found an acetaminophen-mediated decreased expression of Selenbp 2 (fold change of -1.22, although not significant in the multiple comparison test). However, Selenbp 2 did not only respond to acetaminophen treatment, the same decrease was also found after cyclosporin A treatment of primary mouse hepatocytes. Previously, Selenbp 2 was found downregulated in the livers of CCl4-treated mice, a model for liver fibrosis and in ($Abc4^{-}/^{-}$) mice, a model for sclerosing cholangiocytes (Henkel et al., 2006). Moreover, a reduced level of

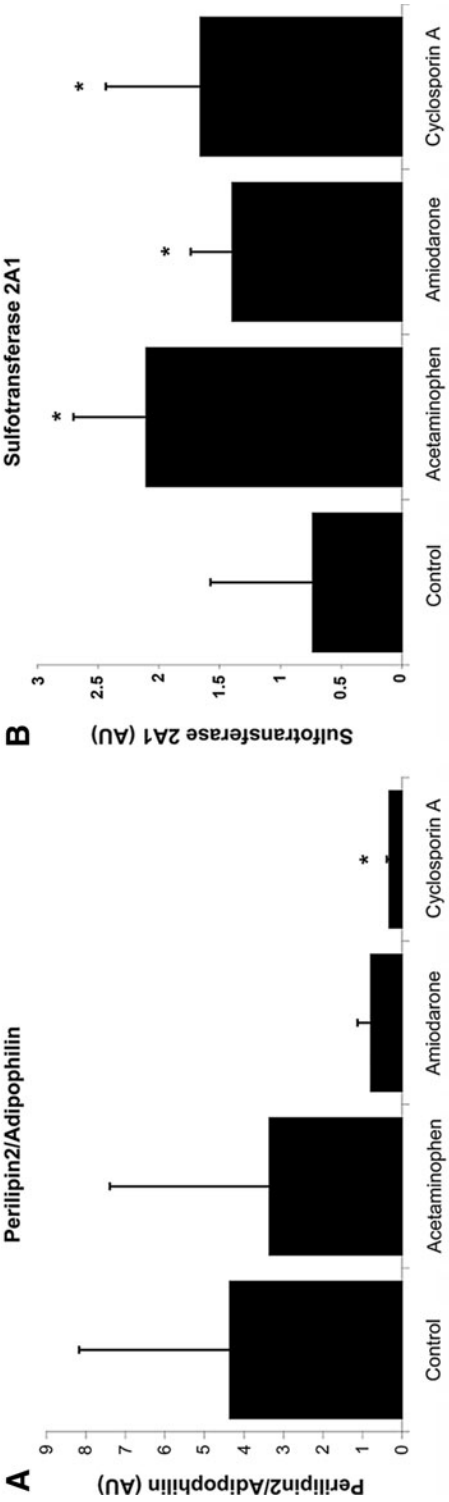

FIG. 5. Expression differences by western blotting of **(A)** perilipin2/adipophilin and **(B)** bile salt sulfotransferase from primary mouse hepatocytes treated with acetaminophen, amiodarone, or cyclosporin A.

selenium in serum and an increased concentration hepatic selenium were earlier already linked to cholestasis (Aaseth et al., 1995, Singh et al., 1992).

Similar to our previous study (Van Summeren et al., 2011) several proteins related to ER-stress were identified. For example, the ER-stress marker 78 kDa glucose-regulated protein was upregulated after cyclosporin A treatment. In addition, the protein disulfide isomerases A3, A4, and A6 were differentially expressed. Notably, the changes in these proteins were all accompanied by transcriptional upregulation. Protein disulfide isomerases are important enzymes for proper protein-folding; for this purpose they work in close collaboration with cyclophilins. Cyclophilins are peptidyl-prolyl-trans isomerases, enzymes that accelerate or slow down steps in the folding of proteins. Moreover, they are involved in the protein quality control in the ER of living cells (Bernasconi et al., 2010). Cyclosporin A is known to be a specific inhibitor of the cyclophilin family. Previously, cyclosporin A was found to translocate cyclophilin B from the ER and promotes its secretion (Price et al., 1994). This leads to a decreased expression of cyclophilin B in the cell and an increased expression of cyclophilin B in the secretome (Lamoureux et al., 2011). Here, we observed a decreased expression of cyclophilin B, while its transcription is upregulated. This was also observed for the other proteins involved in ER-stress and protein-folding. A disturbed ER function may be accompanied by an altered secretion from hepatocytes. Indeed, as in the HepG2 cells, evidence of an altered ER-Golgi transport was found in the primary mouse hepatocytes. Since cyclosporin A induced the differential expression of Sec14l2, this protein is known to both regulate lipid metabolism and trans-Golgi pathways (Curwin et al., 2009). It may regulate cholesterol biosynthesis by increasing the transfer of squalene to a metabolic active pool in the cell (Shibata et al., 2001). In our previous HepG2 study, a remarkable amount of secretory proteins were upregulated in the cellular protein fraction. In primary mouse hepatocytes, we now also found a decreased expression of the serum proteins albumin and serotransferrin. The accumulation of unfolded proteins causing ER stress can cause Ca^{2+} release. High Ca^{2+} concentrations mediate the transfer of an apoptosis signal to mitochondria (Grimm, 2011).

Increased Ca^{2+} concentrations will lead to the opening of the mitochondrial permeability transition pore (MPTP). The MPTP is formed by the interaction of the voltage-dependent anion-selective channel protein (VDAC) with cyclophilin D and adenine nucleotide translocase (Crompton et al., 1998). The opening of the permeability transition pore increases the mitochondrial permeability. This results in an increased osmolar load, mitochondrial swelling, and eventually rupture of the outer membrane, initiating apoptosis. Previously, it was shown that the permeability transition pore is inhibited by cyclosporin A due to its interaction with cyclophilin D, and thereby protecting the cell from apoptosis (Crompton et al., 1998). Here VDAC2 was upregulated after cyclosporin A treatment. Probably this may be an attempt to correct for the inhibition of the MPTP by cyclosporin A.

Mitochondrial dysfunction was also indicated by the differential expression of several enzymes of the urea cycle such as ornithine carbamoyl transferase, arginosuccinate synthetase, and carbamoylphosphate synthase. Ornithine carbamoyl transferase is responsible for the synthesis of

FIG. 6. Venn diagram of the significant differentially expressed proteins in HepG2 and primary mouse hepatocytes. Overlaps contain the number of significant differentially expressed proteins in both systems induced by the used hepatotoxicants.

citrulline out of the substrates ornithine and carbamoylphosphate. In the 2DE proteome map, a significant upregulation of this protein after cyclosporin A treatment was detected, whereas the same condition resulted in a remarkably downregulated expression of the mRNA corresponding to this protein. An altered urea cycle is often seen in patients with liver disease, leading to a reduced capacity to detoxify ammonia resulting in hyperammonemia (Olde Damink, et al., 2009).

Conclusion

In conclusion, using primary mouse hepatocytes, as well as HepG2, we were able to distinguish cyclosporin A from control, as well as acetaminophen- and amiodarone-treated samples. However, just as for HepG2, the proteome of primary mouse hepatocytes did not allow us to make an adequate differentiation between acetaminophen, amiodarone, and the control. We believe this may be due to the decreased expression of certain biotransformation enzymes.

Cyclosporin A induced a different set of deregulated proteins in the two models, however these belong to similar pathways. Again, the differential expression of proteins related to cyclosporin A induced ER stress and the ER-Golgi transport, which may alter vesicle-mediated transport and protein secretion. These similar pathways indicate that cyclosporin A induces a response that is conserved in primary mouse hepatocytes when compared to the HepG2 cells.

Several findings in this study suggest that the differential protein expression pattern seen with cyclosporin A is related to protective mechanisms to cholestasis, such as the expression of Ces1 and phase II detoxifying enzymes. This study does not only show that both models can be used to analyze the cholestatic properties of cyclosporin A, but also indicates the conserved response of primary mouse hepatocytes compared to HepG2 cells.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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Abbreviations Used

2DE = two-dimensional gel electrophoresis
DIGE = differential in gel electrophoresis
DMSO = dimethylsulfoxide
ER = endoplasmic reticulum
HDL = high-density lipoprotein
MPTP = mitochondrial permeability transition pore
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAPQI = N-acetyl *p*-benzoquinoneimine
NFDM = nonfat dry milk powder
PCA = principle component analysis
VDAC = voltage-dependent anion-selective channel protein
VLDL = very low-density lipoprotein