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Bromobenzene-Induced Hepatotoxicity at the Transcriptome Level

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Rats were exposed to three levels of bromobenzene, sampled at 6, 24, and 48 h, and liver gene expression profiles were determined to identify dose and time-related changes. Expression of many genes changed transiently, and dependent on the dose. Few changes were identified after 6 h, but many genes were differentially expressed after 24 h, while after 48 h, only the high dose elicited large effects. Differentially expressed genes were involved in drug metabolism (upregulated GSTs, mEH, NQO1, Mrps, downregulated CYPs, sulfotransferases), oxidative stress (induced HO-1, peroxiredoxin, ferritin), GSH depletion (induced GCS-l, GSTA, GSTM) the acute phase response, and in processes like cholesterol, fatty acid and protein metabolism, and intracellular signaling. Trancriptional regulation via the electrophile and sterol response elements seemed to mediate part of the response to bromobenzene. Recovery of the liver was suggested in response to BB by the altered expression of genes involved in protein synthesis and cytoskeleton rearrangement. Furthermore, after 48 h, rats in the mid dose group showed no toxicity, and gene expression patterns resembled the normal situation. For certain genes (e.g., CYP4A, metallothioneins), intraday variation in expression levels was found, regardless of the treatment. Selected cDNA microarray measurements were confirmed using the specific and sensitive branched DNA signal amplification assay.

Key Words: **toxicogenomics; bromobenzene; transcriptomics; hepatotoxicity; rat; cDNA microarray.**

Bromobenzene (BB), an industrial solvent and an additive in motor oils, causes necrosis in the liver and kidney. The metabolism and toxicity of BB in (rat) liver have been studied in detail (Casini *et al.*, 1985; Lau and Monks, 1988; Miller *et al.*, 1990; Monks *et al.*, 1982; Thor *et al.*, 1981). To enable excretion in urine, BB is subjected to biotransformation in the liver, and metabolites of BB are highly hepatotoxic while secondary metabolites of BB are highly nephrotoxic. Figure 1 schematically depicts the biotransformation of BB in the liver. Upon entrance in the liver, BB is hydrolyzed by cytochrome P450 monooxygenases (CYPs), and inhibitors of CYPs were found to decrease the hepatotoxicity (Madhu *et al.*, 1992). CYP mediated epoxidation yields the highly electrophilic BB 3,4-epoxide. The irreversible binding of this very reactive metabolite to proteins like glutathione S-transferase (GST), liver fatty acid binding protein (L-FABP), and carbonic anhydrase, is highly correlated to pathological effects (Koen *et al.*, 2000). The alternative, more stable, BB 2,3-epoxide was found to covalently bind soluble proteins like hemoglobin (Lau and Zannoni, 1981). Phase II drug metabolizing GSTs catalyze the sequestration of the reactive epoxides through conjugation to glutathione. The levels of glutathione (GSH) conjugates excreted in the bile correlated with the BB-dosage and the hepatotoxic effects (Madhu and Klaassen, 1992). The epoxides are also hydrolyzed by the microsomal epoxide hydrolase and CYPs. The resulting bromophenols can be oxidized to hydroquinones, and conjugated to GSH. At high doses, conjugation to the metabolites depletes the hepatic GSH pool, and the intracellular protection against reactive oxygen species (ROS) and hazardous xenobiotic metabolites is lost. This may lead to a number of secondary events that damage the cell, like lipid peroxidation (Benedetti *et al.*, 1986), ATP depletion (Locke and Brauer, 1991; Wang *et al.*, 1998), mitochondrial dysfunction, energy imbalance, and altered intracellular calcium levels.

Recently, we reported a study where transcriptomics and proteomics were used to investigate BB-induced hepatotoxicity (Heijne *et al.*, 2003). We explored the application of transcriptomics and proteomics in toxicology, and identified proteins that changed specifically 24 h after a single injection of BB. An increased abundance of proteins with lower molecular mass was observed, possibly indicating specific protein degradation. At the same time, a wide spectrum of genes in rat liver was differentially expressed. However, extensive toxicological examinations were omitted and only limited conclusions could be drawn on the mechanism of hepatotoxicity.

A new transcriptomics study was designed to investigate the sequence of events in time in hepatotoxicity, after oral exposure to BB, and to show the dose dependency of the observed effects at the transcriptome level. Measuring the expression of thousands of genes allowed more in-depth investigations in the

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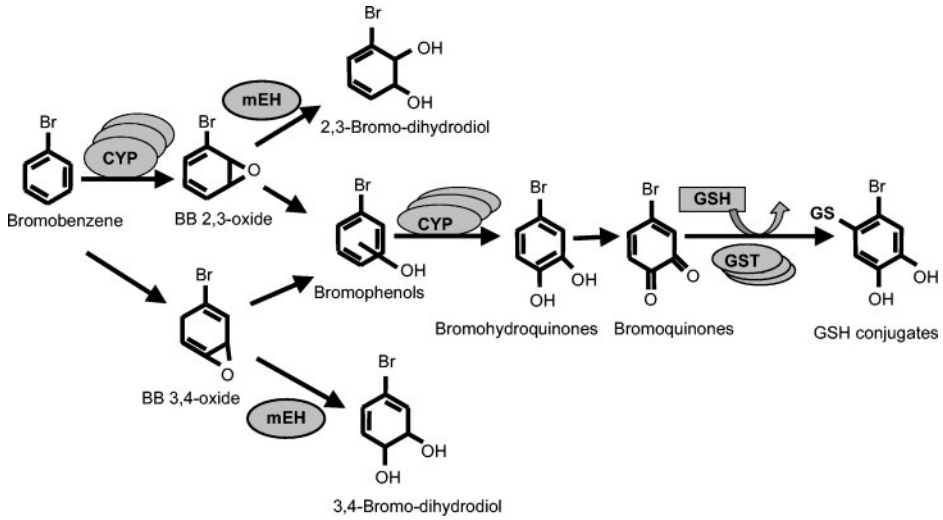


FIG. 1. Mechanism of biotransformation of BB in rat liver. A schematic representation is given of the initial steps in biotransformation of bromobenzene in rat liver.

hepatic changes at the molecular level in response to BB administration. Moreover, we expected to detect changes in gene expression at lower dose levels and earlier time points compared to the routine toxicity examinations. Rats were given BB by po gavage, at three dose levels and liver gene expression profiles were determined 6, 24, and 48 h later. Histopathology and clinical chemistry parameters in plasma were determined, as well as glutathione contents of the liver. Expression of several genes, selected based on the cDNA microarray results, was analyzed in more detail using the branched DNA signal amplification (bDNA) assay.

MATERIALS AND METHODS

Animal treatment and sample preparation. Bromobenzene, 99%, was obtained from Sigma-Aldrich Chemie, GmbH (Steinheim, Germany). The LD_{50} of BB was reported to be approximately 20 mmol/kg body weight for male Crl:Cd rats (Haskell Laboratory for Toxicology and Industrial Medicine, 1981, unreviewed). Three doses of BB (0.5, 2.0, and 5.0 mmol/kg body weight, dissolved in corn oil, 40% v/v) were administered by oral gavage to male Wistar rats (Charles River Deutschland, Sulzfeld, Germany), 10–12 weeks, body weight approximately 200 g. Nonfasted rats were assigned to the groups by randomization, and nine rats per dose group were treated with BB or corn oil, while an additional group contained the untreated controls (UT). Animals were kept under controlled conditions according to international guidelines and national legislation regarding proper care and ethical use of animals. After dosage, rats received water and food *ad libitum,* but no food during the 6 or 16 h before sacrifice. The rats were dosed at 0900 h on day 1. Three rats from each group were sacrificed after 6, 24, and 48 h, blood was collected in heparin tubes and livers were immediately dissected, frozen in liquid nitrogen, and stored at −80°C until further processing. A section of the liver was kept aside in formaline for pathological examination. Blood plasma was isolated for clinical chemistry. Livers were pulverized by mortar and pestle in liquid nitrogen.

GSH and clinical chemistry parameters. Glutathione ([GSH + GSSG] and [GSSG]) levels in liver homogenate were determined spectrophotometrically according to Anderson (1985), and from these values, reduced glutathione, ([GSH]), levels were calculated. Lactate dehydrogenase (LDH), alkaline phosphatase (ALP), ASAT (asparate aminotransferase), ALAT (alanine aminotransferase), bilirubin, cholesterol, phospholipids, triglycerides, glucose, GGT (gamma-gluramyl transpeptidase), creatin, albumin, urea and albumin to globulin (A/G) ratio in plasma were determined using a Hitachi-911 Bioanalyzer, using Boehringer reagents, according to the manufacturer's protocols. One-way analysis of variance (ANOVA) was used to assess the level of statistical significance of the changes.

RNA extraction. Total RNA was extracted from liver homogenate using Trizol (Life Technologies S.A., Merelbeke, Belgium) according to the manufacturer's protocol, and further purified using the RNEasy kit (Qiagen, Westburg B.V., Leusden, Netherlands), including a DNA digestion by RNAse-free DNAseI incubation. RNA was checked for purity and integrity by agarose gel electrophoresis and the concentration was determined spectrophotometrically.

cDNA Microarray preparation and labeling. cDNA microarray preparation was described previously (Heijne *et al.*, 2003). Briefly, about 3000 different sequence-verified rat cDNA fragments were arrayed on glass slides, and control spots were included. A typical labeling reaction was performed using 25 μg of total RNA, using the Cyscribe (Amersham Biosciences, Freiburg, Germany) fluorescence labeling kit. Cy3 or Cy5 fluorophore dUTP nucleotides were directly incorporated in the cDNA during the *in vitro* transcription reaction. RNA was degraded by hydrolysis in NaOH (30 min at 37°C). Labelled cDNA was purified using an Autoseq G-50 (Amersham Biosciences, Freiburg, Germany) chromatography column. Hybridization of labeled cDNA to the slides was performed as described before (Heijne *et al.*, 2003).

Image capture and analysis. Slides were scanned using a (Packard Biosciences) ScanArray Express confocal laser scanner, at wavelength 550 nm (cy3 signal) and 650 nm (cy5). TIFF images were analyzed using Imagene (Biodiscovery Inc., El Segundo, CA USA), and settings were applied to automatically flag weak or negative signals and spots with a nonhomogenous signal. Excel (Microsoft Corporation, Redmond, WA USA) and SAS (SAS, Cary, NC) were used to further process and analyze the data.

Transcriptomics experimental design. In order to compare all samples of individual rats to each other, and to other studies, an external reference sample was used. Thus, for each gene fragment, the amount of mRNA in the sample relative to the amount in the reference was determined. The complete set of hybridizations was duplicated with swapping of the fluorophore incorporation in the sample and reference RNA. The value of this reference RNA has been described before (Heijne *et al.*, 2003). A good correlation was found between the changes in gene expression determined in direct hybridizations and in indirect hybridizations using the reference.

DNA microarray data preprocessing. After image analysis, the local background intensity was subtracted from the signal for each spot. Background

intensities outside the cDNA spots were very low and homogeneous. Control spots and background fluorescence were used to determine a minimal signal to noise ratio threshold value of 1.5 for the two channels. Flagged spots and controls were excluded from further interpretation, as well as genes for which less than 75% of the microarrays yielded an acceptable signal. To account for technical variations introduced during labeling or hybridization, data were normalized assuming that the majority of the transcripts was equally present in both samples. Since a relationship was found between the intensity of the signals and the variation in the ratio of gene, the lowest normalization algorithm, according to Yang and Speed (2002), was applied in SAS software. This procedure fits the expression ratios to an intensity-dependent curve by locally weighted regression. After normalization and logarithm transformation of the ratios tester/reference, a set of about 2700 rat cDNAs was used for further analysis. Excluded values were replaced by 0, and averages were calculated of the logarithms of fold changes between treatment groups.

DNA microarray data analysis. Pairwise comparisons of the expression ratios were made between samples of the different time and dose groups and controls. In these comparisons, untreated and corn oil control samples were considered as one control group, since preliminary analyses revealed only minor changes induced by single oral corn oil dosage. Statistics (two-sided, unpaired *t*-tests) was applied assuming unequal variance and changes were considered significant if the tests resulted in a *p*-value less than 0.01. In the tables, *p*-values were denoted as follows: $***p < 0.001$; $**p < 0.01$. The genes significantly differentially expressed upon treatment were explored in the context of biological mechanisms and pathways. Genes were categorized based on biological processes using literature and gene information databases.

Gene expression measurement by branched DNA signal amplification assay. mRNA levels of microsomal epoxide hydrolase (mEH), heme oxygenase 1(HO-1), NQO1, Mt-1, gamma cysteine ligase catalytic (heavy) chain (or gamma-glutamyl cysteine synthethase) (GCLC), CYP2B1/2, CYP4A2/3, multidrug resistance protein 1 (Mrp1), Mrp2, and Mrp3, and glyceraldehyde phosphate dehydrogenas (GAPDH) were analyzed for all samples individually by the bDNA assay using probes specific to each transcript (Quantigene HV10 kits, Genospectra, www.genospectra.com) as described in Cherrington *et al.* (2002) and Slitt *et al.* (2003). Oligonucleotide probesets that detect NQO1, CYP2B1/2, CYP4A2/3, Mt-1, Mrp1, Mrp2, and Mrp3 were previously described (Cherrington *et al.*, 2002; Hartley and Klaassen, 2000; Li *et al.*, 2002) Oligonucleotide probesets for mEH, HO-1, and GCLC are described in Table 5 (Supplementary Material)

RESULTS

Changes in the liver transcriptome were related to classical toxicological parameters, which showed toxicity only after the highest of three dose levels of BB. Dose-dependent effects of BB at the transcriptome level were analyzed per time point. Additionally, we report intraday variation in gene expression regardless of the treatment. The bDNA assay was used to further investigate changes highlighted by the DNA microarrays.

(Histo)pathology

No significant changes in body weights or macroscopic changes were seen. Neither the livers of the controls nor of the low or mid dose groups displayed pathological aberrations. The livers isolated 24 h after a high dose of BB revealed a patchy appearance and gross lesions, while the livers of rats sacrificed 48 h after a high dose had focal discoloration. Six h after dosing of mid and high levels of BB, relative liver weights were around 10% lower than the controls (ANOVA + Dunnett's test, $p < 0.05$ for high and $p < 0.01$ for mid). At 24 or 48 h, controls as well as low dosed rats had relative liver weights of 75–85% of the rats sacrificed after 6 h. In all the high BB treated rats, a significant increase to around 130% of vehicle controls ($p < 0.01$) was observed. Microscopic examination of the livers showed slight presence of mononuclear cell aggregates and/or necrotic hepatocytes in several rats regardless of the treatment. After 24 and 48 h, pronounced centrilobular necrosis was found in all rats of the high dose groups, with interindividual variation from very slight to very severe necrosis.

Clinical Chemistry

Clinical chemistry parameters in plasma are represented in Figure 2. Bilirubin levels (panel A) in the mid dose group were increased after 24 h, but not after 48 h. In the high dose group, bilirubin levels were slightly up at 6 h, and highly elevated after 24 h and 48 h. Only upon high dose, the rats showed highly elevated alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) levels after 24 h and 48 h

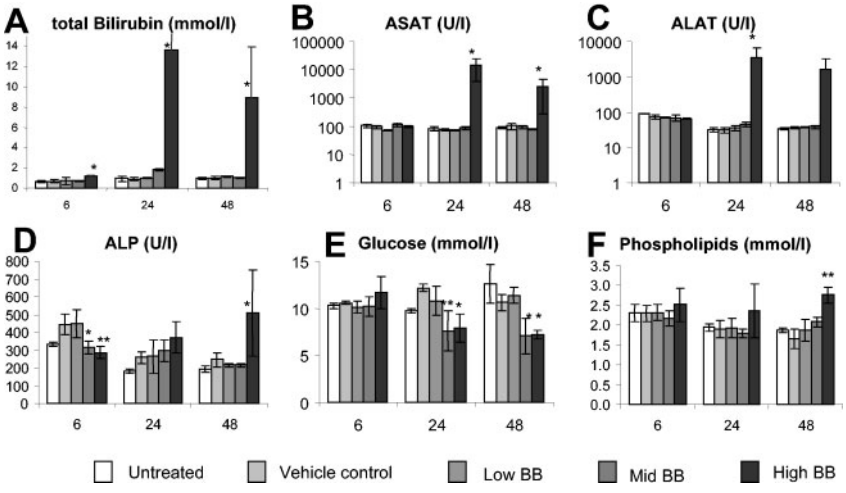


FIG. 2. Clinical chemistry parameters in blood plasma. Measurements of clinical chemistry parameters in plasma. Levels are depicted of bilirubin (panel A), ASAT (panel B) and ALAT (panel C), alkaline phosphatase (ALP, panel D), glucose (panel E), and phospholipids (F). Average levels of the treatment groups ± SD are represented. Statistical significance is denoted with ** for a *p*-value < 0.01; *** for a *p*-value < 0.001.

(panels B and C). Plasma lactate dehydrogenase (LDH, not shown) activity was increased 100-fold 24 h after the high dose, but showed normal levels at 48 h. Alkaline phosphatase (ALP; panel D) activity in plasma gradually increased with dose at 24 h (not significant), and was elevated 48 h after the high dose. Plasma glucose (panel E) decreased equally by mid and high dose after 24 and 48 h. Cholesterol (not statistically significant [n.s.]) and phospholipid levels (panel F) in the high dose group were increased at 6 h and remained elevated. The creatin content decreased upon the high dose after 6 h (n.s.). Regardless of the treatment, urea levels were higher and triglyceride levels lower at 24 and 48 h compared to 6 h. No significant changes were observed in plasma levels of gamma-glutamyl transpeptidase, total protein, albumin, and the albumin to globulin ratio (A/G).

Glutathione Depletion

Hepatic reduced GSH levels, after correction for oxidized disulfide of glutathione (GSSG), are shown in Figure 3. An intraday variation in the concentration of GSH was observed in control rats. More GSH was present at 6 h compared to the 24 h or 48 h time point. The low dose of BB caused a slight decrease of GSH levels after 6 h, whereas both the mid and high BB doses depleted GSH to ~25% of control levels. After 24 h, GSH levels in the mid dose group increased significantly to ~125% of controls, and remained elevated 48 h after dosage.

Gene Expression

Pairwise comparisons of the gene expression levels were made between treated and control groups. After 6 h, only few changes could be identified, whereas many genes changed in expression 24 and 48 h after dosage. Overlap of the genes differentially expressed upon BB treatment at all time points

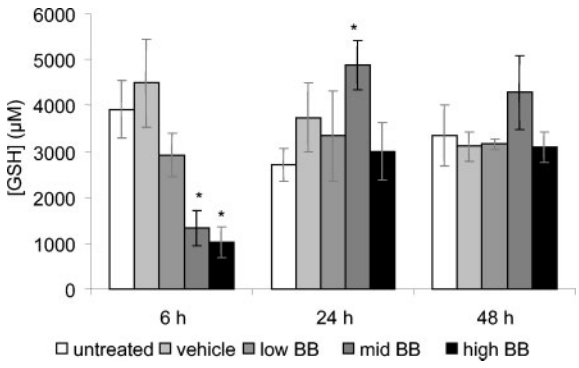


FIG. 3. Hepatic intracellular glutathione levels. Intracellular reduced glutathione (GSH) levels were determined of rat liver homogenate of untreated, corn oil, and bromobenzene treated rats, 6, 24, and 48 h after a single dosage. Average levels of the treatment groups ± SD are represented. White bars represent samples taken 6 h after dosage, grey bars 24 h, and black bars represent samples taken 48 h after dosage. UT: untreated; CO: corn oil controls; L, M, and H denote the low, mid, and high dose of bromobenzene. Statistical significance is denoted with * for a p-value < 0.005.

was observed, although many genes were altered with statistical significance only at one time point. The individual gene expression profiles of all the BB-treated rats sacrificed after 24 h were recorded in duplicate. Many genes were regulated after a high (176 genes) or mid dose of BB (41 genes), whereas a low dose induced changes that were significant in only 6 genes ($p < 0.01$). The majority of the genes significantly regulated 24 h after the mid dose were also regulated after the high dose, whereas most of the genes that were changed upon the low dose were altered uniquely in this group.

Six h

Table 1 lists the genes that were significantly differentially expressed after 6 h in the BB dosed rats. A marked (2–8 fold) increase in gene expression was observed for several metallothioneins (Mt). Both the flavin-containing mono-oxygenases FMO1 and FMO3 were upregulated by the high dose, as well as cathepsin L and ASAT. The enzymes cysteine dioxygenase 1 and betaine homocysteine methyltransferase were upregulated. A two-fold upregulation was found for mRNA encoding Rho-interacting protein 3. Genes downregulated by the high dose include LDH B, tubulin, and cholesterol metabolism enzymes farnesyl diphosphate synthase and farnesyl diphosphate farnesyl transferase 1, "sterol-C4-methyl oxidase-like," HMG-CoA synthase 1. Several genes, HO-1, TIMP1, aflatoxin B1 aldehyde reductase (AFAR), and serine protease 15, changed significantly with the mid or the low dose after 6 h but not with the high dose. Three genes were down regulated by both the mid and high dose, namely the "EST similar to GSTP," HSP70, and calpain.

Twenty-four h

Table 2, in Supplementary Material, lists the subset of genes that changed significantly 24 h after BB, in the high dosed rats. Genes were categorized according to biological processes. Among many others, GSTA and GSTM, mEH, AFAR, ferritins, peroxiredoxin, transketolase1, GAPDH, NQO1, proteasome subunit alpha1, and ALP were markedly elevated upon mid and high dose. The genes encoding "EST similar to human phosphodiesterase (cAMP specific)" and the alpha(1)-inhibitor 3 were markedly down regulated by all doses of BB, while the glucocorticoid receptor was mildly but significanly downregulated by all doses (not shown). Decreased upon mid and high dose were the genes encoding cysteine dioxygenase, asialoglycoprotein receptor 2, 14-3-3 protein (Ywhaz), "EST similar to GSTP," sulfotransferase 2 and the transcription factor "core promotor element binding protein" (C/EBP).

Forty-eight h

The differential expression of genes 48 h after the high concentration of BB was similar to the expression pattern after 24 h (Table 3, in Supplementary Material) Many of these 175 genes were also differentially expressed after 24 h, although

TABLE 1
Gene Expression Changes 6 h after Bromobenzene Dosage

Category	Gene name	ACC #	Low	Mid	High
Ox. stress					
	Heme oxygenase (HO-1)	AA874884	0.76	2.32**	0.57
	Tissue inhibitor of metalloproteinase 1 (TIMP1)	AA957593	1.14	1.92**	0.51
Acute Phase; Ox. stress					
	Metallothionein (Mt)	AA859399	0.98	3.52	3.01***
	Metallothionein (Mt)	AA900218	0.71	3.06	2.42***
	High sim to METALLOTHIONEIN-II (MT-II) [Rat]	AA924281	0.91	1.88	2.15***
	Weak sim to METALLOTHIONEIN-I (MT-I) [Rat]	AA819204	0.43	1.04	1.19**
Acute Phase?					
	Nuclear protein 1	AI070183	0.22	−0.42	−0.58**
Aminoacid metab.					
	Aspartate aminotransferase (ASAT), GOT1	AA900928	−0.28	−0.32	0.82**
Cholest. metab.					
	Farnesyl diphosphate farnesyl transferase 1	AA818927	−0.34	−0.40	−0.71**
	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	AA924800	−0.26	−0.21	−0.83**
	Farensyl diphosphate synthase	AA859192	−0.30	−0.79	−0.87**
Cholest. metab.?					
	Sterol-C4-methyl oxidase-like	AA859607	−0.21**	−0.08	−0.31**
Cysteine metab.					
	Cytosolic cysteine dioxygenase 1	AA818579	−0.30	0.13	0.67**
	Betaine-homocysteine methyltransferase	AA901407	0.08	−0.07	0.61**
Drug metab.					
	Flavin-containing monooxygenase 3 (FMO3)	AA964011	−0.03	−0.18	0.87**
	Flavin-containing monooxygenase 1 (FMO1)	AA860001	−0.03	0.14	0.46**
	Cytochrome P450 4A3 (CYP4A3)	AA924591	0.33	0.38**	0.19
	Cytochrome P450, 2b19 (CYP2B19)	AA818412	−0.63**	−0.21	0.08
	Sulfotransferase family, 2A1	AA819605	−0.87**	−0.43	−0.33
Drug metab; GSH					
	Glutathione S-transferase 1 (theta) (GSTT1)	AI044236	−0.13	−0.26**	−0.03
	Weak sim to GST P (GST 7-7) (class-pi) [Rat]	AA819810	−0.40	−0.64**	−0.56**
Drug metab.					
	Aflatoxin B1 aldehyde reductase (AFAR)	AA923966	1.09	1.23**	0.07
Heat shock					
	heat shock 70kD protein 5 (HSP70)	AI453996	−0.27	−0.86**	−0.70**
Immunoresponse					
	ig delta heavy chain constant region	AA964201	0.09	0.15	0.26**
	MHC class I	AA818887	−0.28	−0.22	−0.53**
LDH					
	Lactate dehydrogenase B (LDH)	AA819821	−0.32	−0.27	−0.59**
Proteolysis					
	Cathepsin L	NA	−0.18	−0.08	0.59**
	Protease, serine, 15	AI070052	0.53**	0.39	0.24
	Weak sim to ubiquitin conjugating enzyme [Rat]	AA818770	0.28**	0.10	−0.01
	High sim to leucine aminopeptidase [Mouse]	AA858780	−0.35	−0.41	−0.48**
Proteolysis, structure					
	calpain 1	AA901002	0.06	−0.56**	−0.34**
Proteolysis?					
	Ubiquitin D	AI030354	−0.32	−0.34	−0.44**
Ribosome					
	Ribosomal protein L22	AA924274	−0.16	−0.18	0.64**
	Ribosomal protein L28	NA	0.30	0.44**	0.13
	Ribosomal protein L35	AA925200	−0.05	0.38	−0.30**
Structure					
	Rho interacting protein 3	AA924848	−0.13	0.09	1.01**
	Unconventional myosin from rat 3	AA818082	−0.77**	−0.27	0.10
	Actinin alpha 4	AI712704	−0.21	−0.21**	−0.11
	beta-tubulin T beta15	AA899219	−0.31	−0.20	−0.56**
Transcrip. factor					
	Transforming growth factor beta stimulated clone 22	AI137902	0.10	−0.64**	−0.30
Transport					
	Group-specific component (vitamin D-binding protein)	AA818706	0.24	0.35**	−0.03
Signal transd.					
	14-3-3- protein Ywhaq	AA858957	−0.02	−0.33	−0.34**
Signal transd.?					
	S6 kinase	AA900032	0.10	−0.12	0.36**
Other					
	High sim to Suc.-CoA:3-ketoacid-coA transf.[Hum.]	AA819087	−0.46	−0.23	−0.72**

Note. The subset of hepatic genes that were significantly differentially expressed 6 h after the BB administration compared to the controls. Genes were categorized according to biological mechanism or pathways. The log (base 2) average fold change and the *p*-value for the three dose levels are listed. A threshold was chosen for display reasons which was a log (base2) fold change > 0.5 or < -0.5 in any of the dose groups compared to the controls. ACC#, Genbank Accession number; metab, metabolism.

** $p < 0.01$.

*** $p < 0.001$.

frequently the difference was less marked and not statistically significant. Only two genes, interferon-inducible transmembrane protein and steroid sulfatase were found to be subtly but significantly differentially expressed at the low BB dose. The mid dose slightly induced three genes, also induced by the high dose.

Intraday Variation

The control rats were used to analyze the intraday variation in liver gene expression as well as in other parameters. Intraday differences were observed in GSH content and relative liver weights as well as in levels of parameters in plasma. Triglyceride levels were lower at 24 h (and 48 h) than at 6 h. This was also the case for ALP, phospholipids, cholesterol, and urea Also at the gene expression level, the 6 h time point was found to be clearly distinct from the 24 and 48 h time points. Profiles obtained from untreated rats were very similar to those obtained from corn oil treated rats. No genes were recognized with a marked change in expression between 24 and 48 h in the controls. Table 4 (Supplementary Material) shows genes in which expression changed significantly from 6 to 24 h in controls. The most distinctly higher expressed genes were metallothioneins. Moreover, various CYP isozymes (CYP4A1 and CYP4A2/3), arginosuccinate lyase, cathepsin L, and various genes involved in fatty acid metabolism were higher expressed at 24 h than at the 6 h time point. Genes in which expression was lower after 24 h compared to 6 h included several GSTs, transferrin, tubulin, lysyl hydroxylase, and GSH peroxidase.

Confirmation of Gene Expression Changes

cDNA microarrays results for selected genes were analyzed for all rat samples using the bDNA assay (Fig. 4). This method was shown to very specifically determine mRNA levels in a wide concentration range. The gene expression levels for mEH, GAPDH, HO-1, Mt-1, NQO1, and CYPs in the bDNA assay largely overlapped with the cDNA microarray results. For example, between both methods, a coefficient of correlation of 0.94, 0.89 and 0.86 was found for the individual rats' levels of mEH, HO-1, and Mt-1, respectively. Also, genes were analyzed that were not (conclusively) measured in the microarrays. We hypothesized these genes (Mrp1, Mrp2 or Mrp3, GCLC) could be modulated based on other changes observed using the microarrays, for instance indicating EpRE-mediated transcriptional regulation. GAPDH, which is frequently regarded as a so-called housekeeping gene with stable expression levels, was measured using both the microarrays and the bDNA assay. By both methods, GAPDH was found to be upregulated more than two-fold at high dose levels after 24 h.

DISCUSSION

The dose and time related effects of acute hepatotoxicity induced by BB were analyzed at the gene expression level. Hepatic centrilobular necrosis was found after 24 h in rats at the high dose, but not at lower doses. Also clinical chemistry parameters detected hepatocellular damage only after 24 h in the high dose group. Significant changes in expression of genes upon the high, mid and even the low dose were identified, especially after 24 h. The large part of the genes identified to change significantly upon the high dose also responded to the mid dose, although frequently not as pronounced or not with the same statistical significance. Thus, a dose-relationship can be observed in transcriptomics experiments, both in the number of genes that change and the magnitude of the changes. In time, the mid dose elicited most effects after 24 h, while after 48 h, hardly any change persisted. In contrast, in the high dose group, those effects identified after 24 h persisted after 48 h.

Paradoxically, several genes including mEH, AFAR, HO-1, and TIMP1 were markedly upregulated by the low and mid dose at 6 h, but not by the high dose. At later stages, these genes were highly upregulated by BB in a dose-dependent manner. The effects measured by cDNA microarrays were confirmed using the bDNA assay. The initial lack of response with the high dose contradicts the dose-dependency usually assumed in toxicology. No explanation was found for this phenomenon, and no such observations were found in literature.

Pathways and Mechanisms

Genes with statistically significant differential expression upon BB administration were categorized according to biological processes in the cell, putatively relevant in BB-induced hepatotoxicity. The most relevant changes in BB-induced hepatotoxicity were schematically displayed in Figure 5.

Drug Metabolism

The strong induction of transcripts encoding GSTA and GSTM stresses the importance of GSH conjugation in the biotransformation of BB. The upregulation of mEH messenger RNA (mRNA) levels by BB is coherent with its role; the hydrolysis of epoxide BB intermediates, enabling their excretion. The epoxides are among the most reactive BB-metabolites and their hydrolysis is crucial in the hepatic detoxification. The mEH is also induced by xenobiotics like phenobarbital, *trans*-stilbene oxide, and Aroclor 1254 (Oesh and Arand, 1999). BDNA assays revealed about two-fold increased expression levels as early as 6 h after the low dose. Mid or high doses elevated mEH expression after 24 h, while after 48 h, mEH was pronouncedly upregulated only in the high dose rats. Similar to mEH, induction of NQO1 was identified using the bDNA assays. NAD(P)H quinone oxidoreductase (NQO1) is an enzyme with a role in protection of cells from oxidative stress, cytotoxicity, and mutagenicity of quinones, which are also formed in the biotransformation of BB. The induced AFAR, and aldo-ketoreductases exert putative roles in metabolism of xenobiotics and products from lipid peroxidation like

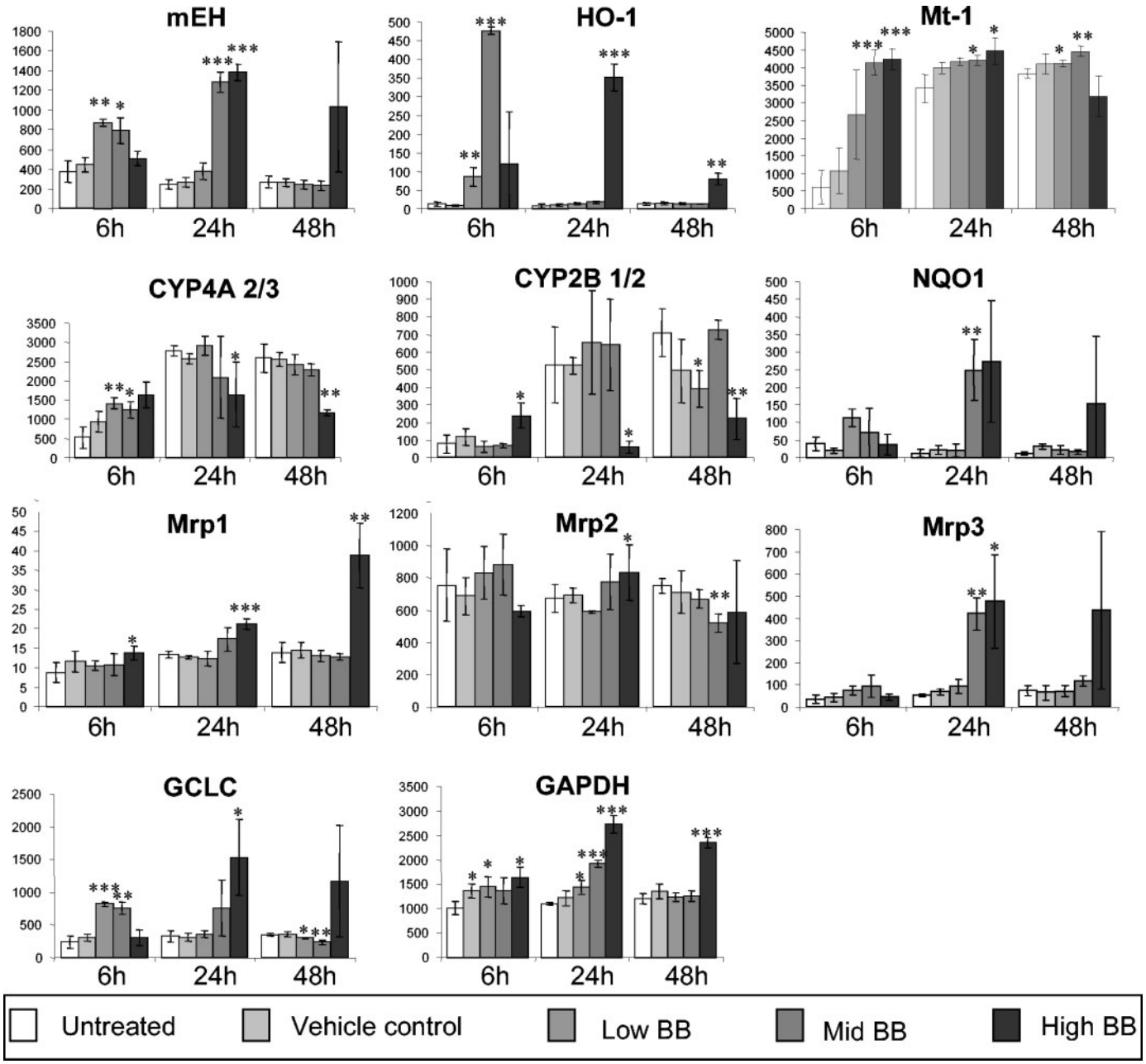
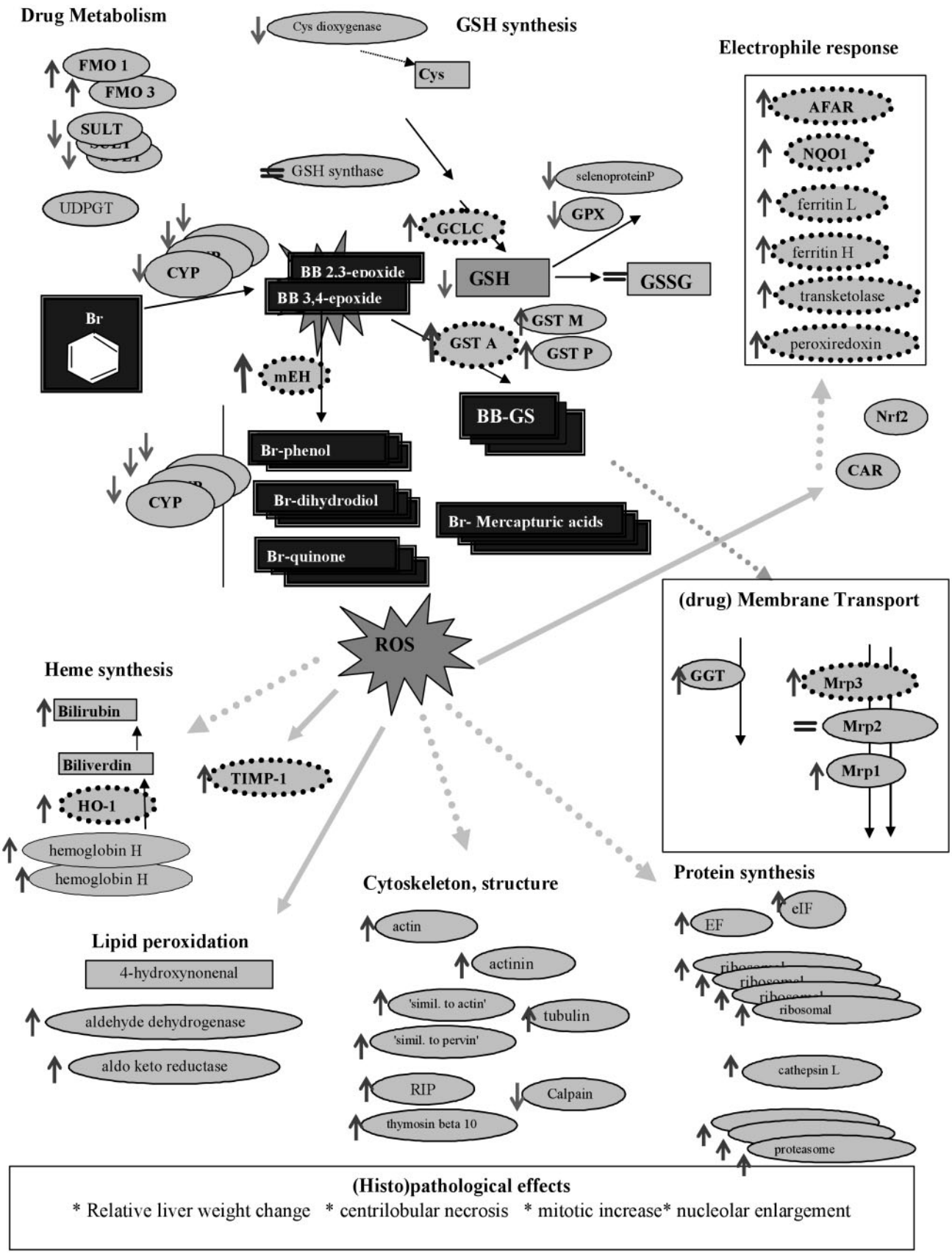


FIG. 4. Hepatic gene expression levels as measured by the Branched DNA signal amplification assay. Average levels of the treatment groups ± SD are represented. White bars represent samples taken 6 h after dosage, grey bars 24 h, and black bars represent samples taken 48 h after dosage. UT: untreated; CO: corn oil controls; L, M, and H denote the low, mid, and high dose of bromobenzene. Statistical significance is denoted with **for a p-value < 0.01; ***for a p-value < 0.001.

4-hydroxy-2-nonenal. The early induction of FMOs has possible implications in the biotransformation of BB as well. FMOs have a broad substrate specificity and their functions partly overlap with CYPs. Induction of FMO1 was found in rat liver by the polycyclic aromatic hydrocarbon, 3-methylcholanthrene (Chung *et al.*, 1997). Different CYP (2B1/2, 2E1, 2A2C39, 4A2/3, 4A10, 2C12, 2C23) and sulfotransferases were down regulated and remained lower after 24 h. The bDNA assay confirmed specific changes in expression levels of the CYP2B 1/2 and CYP4A 2/3 isoforms. At 6 h, BB slightly increased CYP2B and CYP4A expression, while after 24 and 48 h, expression was reduced upon high BB treatment. Moreover, intraday variation in expression was observed in the controls. The decreased gene expression of CYPs could diminish the bioactivation of BB to harmful metabolites. Expression of the multidrug resistance proteins Mrp1, Mrp2, and Mrp3 was



measured with the bDNA assay. The change in expression of Mrp1, Mrp2, or Mrp3 was not measured in the microarrays, but Mrp3 induction was hypothesized from the suggested EpRE-mediated induction of several genes. A pronounced induction of Mrp3 gene expression was observed 24 h after high BB treatment, whereas the Mrp2 expression did not change. Mrp1 expression levels were very low in the liver, but an induction was observed by high BB after 24 h. Substrates for Mrp-facilitated export across the membranes include glucuronide, sulfate, and GSH conjuates. The induction of Mrps by BB could enhance the biliary excretion of conjugated BB metabolites, as found for acetaminophen metabolites (Slitt *et al.*. 2003; Xiong *et al.*, 2002). The concurrent increased hydrolysis of the epoxides, GSH conjugation and clearance of the harmful BB metabolites may provide the liver with an effective mechanism of detoxification.

GSH Metabolism

GSH is believed to be of crucial importance in the detoxification of xenobiotics like BB. A dose-dependent lowering of the liver GSH levels was observed 6 h after BB administration. Compared to our previous study with ip injection of BB (Heijne *et al.*, 2003), total GSH depletion was not observed in this study, 24 h after an oral administration. On the contrary, after 24 h GSH concentration was higher upon BB administration than in the controls. The increased level of GSH after the initial depletion has been suggested as a recovery mechanism (Chakrabarti, 1991). Also, a significant upregulation of GSTA and GSTM was found after 24 h even by the low dose. The rate-limiting enzyme in GSH synthesis is GCLC, transcriptionally regulated by electrophile response element (EpRE). Using the bDNA assay, we detected a pronounced upregulation of GCLC as early as 6 h after the low dose (Fig. 6). Recently, we also found GSH synthase protein to be induced by BB (Heijne *et al.*, 2003). The upregulation of the enzymes in GSH metabolism can be explained as an adaptive response to restore the depleted GSH levels in liver.

Oxidative Stress

Highly reactive metabolites such as formed from BB can induce oxidative stress. Additionally, the depletion of GSH diminishes the intracellular protective mechanism against ROS and electrophilic metabolites The early induction of key markers, the mRNA levels of HO-1 and TIMP1 suggests the induction of oxidative stress by BB. After 24 h, HO-1 mRNA levels were elevated only in the high dose group, while returned to normal in the mid dose group. HO-1 catalyzes the degradation of heme to CO_2 and biliverdin, which is subsequently catabolized to bilirubin. Bilirubin may serve as an intracellular antioxidant. In line with this, we observed bilirubin levels in plasma to be slightly increased in the high dose group at 6 h, while largely increased after 24 h. Also peroxiredoxin 1 and the ferritin light and heavy subunit transcripts were elevated significantly by BB. Ferritins sequester free iron molecules thus preventing formation of ROS, hydroxyl radicals, through the Fenton reaction. Peroxiredoxins are antioxidant enzymes with a role in signal transduction. Metallothionein transcripts were highly induced at 6 h after all doses of BB. At 24 and 48 h an elevation of the Mt mRNA levels was observed independent of the treatment. Metallothioneins are cysteine-rich proteins that function in the sequestration of the metals Cu^{2+}, Cd^{2+}, Zn^{2+}, while also ROS can be scavenged. The BB-induced rapid increase of Mt mRNA corresponds with reported 40% increased protein concentrations in the liver and kidneys 6 h after BB (Wong and Klaassen, 1981; Szymanska *et al.*, 1991). The protein Vdup1 was induced and has been reported to interact with thioredoxin and to be associated with oxidative stress (Wang *et al.*, 2002). GSH peroxidase, selenoprotein P, and synaptojanin 2, genes possibly related to oxidative stress, were down regulated. GAPDH was induced more than two-fold 24 h after the high dose of BB. GAPDH has been reported to be regulated by hypoxia and induced by insulin and glutamine. Responsive elements for hypoxia-inducible factor, and C/EBP have been identified in the GAPDH gene promotor (Claeyssens *et al.*, 2003; Graven *et al.*, 1999; Rolland *et al.*, 1995); The induction of GAPDH might be in concordance with its function in glycolysis, upregulated to meet the energy requirements of the regenerating liver.

Acute Phase Response

The acute phase response is elicited by various types of stress like mechanical damage and inflammation (Ramadori and Christ, 1999; Suffredini *et al.*, 1999). Especially in the liver, changes in many genes and proteins provide a protective response and re-establish cellular homeostasis. We previously identified that the acute phase response was elicited upon an intraperitoneal administration of BB (Heijne *et al.*, 2003). Present experiments showed that also upon oral gavage, BB was able to elicit changes in transcript levels of many acute phase proteins in liver. Orosomucoid 1 (former AGP), cyto-

FIG. 5. Schematic representation of the most prominent changes in bromobenzene-induced hepatotoxicity. The biotransformation of BB in rat liver, and biological processes relevant to the hepatic response to BB are shown. Rectangular boxes represent the metabolites formed from BB in biotransformation. Filled circular boxes represent genes, grey rectangular boxes represent metabolites or small molecules. An arrow is pointing up when the gene is upregulated by BB and an arrow is pointing down when a gene is downregulated by BB. Circular boxes with dashed lines are shown for the genes known to be transcriptionally regulated by an electrophile response element (EpRE). Oxidative stress induced by the reactive metabolites of BB plays a central role in the response at the gene expression level.

keratin-18, and apolipoprotein A1 were induced. Negative acute phasetranscripts including alpha-1-inhibitor 3 and pre-alpha-inhibitor heavy chain 3, serine protease inhibitor, complement component 1 and 4, L-FABP 1 and fibrinogen B were down regulated by BB.

Fatty Acid and Cholesterol Metabolism

Plasma levels of cholesterol increased upon high dose after 6 h and remained elevated at 24 and 48 h. Several enzymes involved in cholesterol metabolism were significantly and dose-dependently down regulated after 6 h by BB. Cholesterol is biosynthesized from acetyl-CoA, the product of fatty acid degradation, and enzymes in both pathways are down regulated by BB, including HMG-CoA synthase, Acetyl-CoA acetyltransferase 1, hydroxylacyl-CoA dehydrogenase, acyl-coA oxidase, trifunctional protein. Many of the genes coordinately downregulated are transcriptionally induced through binding of the sterol responsive element binding protein (SREBP) to a sterol-responsive element in the upstream DNA sequence of those genes (Osborne, 2000; Shimano, 2002). Cholesterol levels play a role in the regulation of the SREBP pathway. The downregulation of the fatty acid and cholesterol metabolism could be due to the requirement of energy for these processes. In this situation of distress, the cell might have to dedicate all energy supplies to cope with the toxicity induced by BB, and restore homeostasis. Energy-requiring processes should be down regulated, while energy-generating processes are upregulated. In line with this, the decrease of plasma glucose levels could be ascribed to increased catabolism of glucose in the glycolysis.

Protein Synthesis and Proteolysis

Genes involved in protein synthesis, including many ribosomal subunits, eukaryotic initiation factors, and elongation factor, were overexpressed one day after dosage. Furthermore, an induction was observed for several components of the proteasome and the proteolytic enzyme cathepsin L. Dose and time related changes in gene expression were observed for serine protease, dipeptidyl peptidase, polyubiquitin and calpain. The histopathologically observed slight nucleolar enlargement and the mitotic increase in the BB-treated livers correspond with the induction of proteins required for transcription and translation.

Structure and Cytoskeleton

Genes encoding cell-structure proteins increased upon the high dose of BB. Stongly upregulated were actin and "weakly similar to pervin," a protein with high homology to a human cytoskeleton-interacting protein. Also actinin, cortactin, "EST highly similar to actin," keratin, dynein, tubulins, and others were differentially expressed. Thymosin beta-4 is an actin binding protein and is upregulated 48 h after a high dose of BB. Rho-interacting protein is involved in cytoskeleton rearrangement, and increases after 6 h. Oxidative modifications of the microfilaments are suggested to cause cytotoxicity in the form of blebs on plasma membranes, when polymerized actin is disrupted by oxidation of its sulfhydryl groups. Upon rupture of the blebs, cellular ion gradients and intracellular components are lost, leading to necrotic cell death. The change of cellular calcium (Ca^{2+}) levels may play a role in the cytotoxicity. Increasing Ca^{2+} levels promote dissociation of actin from α-actinin, and activation of the calpain protease, which cleaves actin-binding proteins. When the anchoring of the cytoskeleton to the plasma membrane is disturbed, membrane blebbing may occur (Boelsterli, 2003; Dalle-Donne *et al.*, 2001). BB caused down regulation of calpain gene expression after 6 h. The induction of both the protein synthesis and the cell-structure genes suggests that enhanced protein synthesis and/or proliferation occur, which aids in hepatic tissue remodelling and recovery after BB-induced hepatocellular injury. Corroborating evidence is found in the histopathological observation of a mitotic increase and nucleolar enlargement in the hepatocytes upon high BB dosage.

Recovery

The gene expression profiles obtained from livers isolated 48 h after a mid BB dose resembled the profiles of the controls and the low dose livers. We suggest that the mid dose still effectuates a marked response, especially detectable 24 h after oral ingestion of BB, but is not high enough to induce irreversible damage detectable by histopathology or clinical chemistry. This suggests that the rats in the mid dose group did not suffer, or recovered from the toxic stimulus. From the time-series, it was clear that most pronounced changes in gene expression were observed 24 h after BB administration. Previously, Lind and Gandolfi (1999) suggested that irreversible changes occurred after 24 h, when the limited centrilobular lesions progressed to a more widespread pattern, and DMSO could no longer attenuate the BB-induced necrosis.

Coordinate Expression Mediated by the Electrophile Response Element

The coordinate induction of HO-1, ferritins as well as GSTA, and NQO1 by BB is consistent with the reported presence of an EpRE, formerly named antioxidant response element (ARE), in those genes (Friling *et al.*, 1990; Kong *et al.*, 2001; Tsuji *et al.*, 2000). Rat GSTA, NQO1, and ferritin were known to be transcriptionally regulated by binding of Nrf2 to this EpRE. More Nrf2-regulated genes were identified in mice upon induction by the isothiocyanate sulforaphane (Thimmulappa *et al.*, 2002). These included AFAR, glucose-6-phosphate dehydrogenase, carboxylesterase, transketolase, and aldehyde dehydrogenase. In our studies in rats, BB induced HO-1, ferritins, GSTA, GSTM, and NQO1, concurrently with TIMP1, AFAR, GSTM, peroxiredoxin1, aldo-keto reductase,

transketolase, and also Mrp3. The rat Mrp3 was recently found to be induced by CAR and EpRE activators in liver (Cherrington *et al.*, 2002). The presence of the EpRE in rat AFAR, as suggested by our data, was confirmed recently (Ellis *et al.*, 2003).

Intraday Variation in Gene Expression

Control rats sacrificed 6 h after the start of the study had higher relative liver weights and also the levels of GSH differed. Urea, triglycerides, and phospholipids levels in plasma varied on an intraday basis. These changes were accompanied by altered liver gene expression. Intraday variation of GSH levels (and of other plasma parameters) might not be a negligible effect on the outcome of pharmacology and toxicity studies. Our results show that also expression levels of certain genes change considerably during the day.

Interstudy Comparison and Route of Administration

Previously, we reported rat liver genes and proteins with altered expression upon ip administration of BB (Heijne *et al.*, 2003). The majority of the genes that changed in the previous study with ip administration of BB again was identified to change in the present study with oral administration of BB. The overlap between the two independent studies demonstrates the robustness of the methods and confirms that our data analysis approach did not allow the introduction of many false positives, frequently raised as a point of concern for cDNA microarray experiments. We conclude that with both routes of administration, ip and po, the same hepatic response was induced at the transcriptome level. The ip injection of corn oil induced some subtle effects, while changes induced by the oral administration of corn oil were not readily detected.

Concluding Remarks

In summary, gene expression measurements in liver 6, 24, and 48 h after dosage of several doses of BB yielded a more comprehensive insight into different cellular pathways that are activated when rats are given BB, leading to hepatotoxicity. Results expanded the findings of our earlier experiments (Heijne *et al.*, 2003). Many changes were in line with the observations from routine toxicological assessments, while also new hypotheses on mechanisms of BB-induced hepatotoxicity were postulated. Recovery of the liver was suggested in response to BB with the altered expression of genes involved in protein synthesis and cytoskeleton rearrangement. After 48 h, the rats in the mid dose group showed no signs of toxicity, concurrent with a gene expression patterns that largely resembled the controls. We identified genes responding to dose levels below 5.0 mmol/kg BW, with some genes responding to oral administration of as low as 0.5 mmol/kg. Thus, we were able to detect significant effects at 2 to 10-fold lower doses with transcriptomics compared to clinical chemistry or histopathology. Genes that could serve as early biomarkers of hepatotoxicity at lower BB exposure were revealed, such as HO-1, mEH, AFAR, and Mt-1. A sample of results from the cDNA microarrays were confirmed by the bDNA assay. Future research will have to establish further whether the changes in gene expression are adverse or protective, and whether they are reversible or irreversible effects.

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