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Combination of 'omics' data to investigate the mechanism(s) of hydrazine-induced hepatotoxicity in rats and to identify potential biomarkers

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To gain novel insight into the molecular mechanisms underlying hydrazine-induced hepatotoxicity, mRNAs, proteins and endogenous metabolites were identified that were altered in rats treated with hydrazine compared with untreated controls. These changes were resolved in a combined genomics, proteomics and metabonomics study. Sprague–Dawley rats were assigned to three treatment groups with 10 animals per group and given a single oral dose of vehicle, 30 or 90 mg kg^{-1} hydrazine, respectively. RNA was extracted from rat liver 48 h post-dosing and transcribed into cDNA. The abundance of mRNA was investigated on cDNA microarrays containing 699 rat-specific genes involved in toxic responses. In addition, proteins from rat liver samples (48 and 120/168 h post-dosing) were resolved by two-dimensional differential gel electrophoresis and proteins with changed expression levels after hydrazine treatment were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry peptide mass fingerprinting. To elucidate how regulation was reflected in biochemical pathways, endogenous metabolites were measured in serum samples collected 48 h post-dosing by 600-MHz ^{1}H-NMR. In summary, a single dose of hydrazine caused gene, protein and metabolite changes, which can be related to glucose metabolism, lipid metabolism and oxidative stress. These findings support known effects of hydrazine toxicity and provide potential new biomarkers of hydrazine-induced toxicity.

Keywords: hydrazine, proteomics, genomics, metabonomics, glucose metabolism, lipid metabolism, oxidative stress.

Introduction

Novel 'omics' techniques such as genomics (De Backer *et al.* 2002), proteomics (Tyers and Mann 2003) and metabonomics (Nicholson *et al.* 2002) are promising tools for elucidating molecular mechanisms of toxicity and to identify biomarkers with the potential to be developed as markers that can be monitored in clinical trials of drug development. The high expectations of these technologies are driven mainly by the completion of the human genome, and the rapid progress in sequencing the genome of animal species used in toxicological testing, e.g. rat and pig.

The three different technologies each have their advantages. Genomics or gene expression analysis allows the simultaneous study of the mRNA levels of thousands of genes. Proteomics is the large-scale identification and characterization of proteins by, e.g. two-dimensional (2D) gel electrophoresis and mass spectrometry. The complexity of the proteome far exceeds the complexity of the genome due to post-translational modification, such as glycosylation (Packer *et al.* 2002),

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phosphorylation (Bykova *et al.* 2003), protein cleavage, etc. Identification of these modifications is very important since they affect stability and activity of the proteins, e.g. protein half-life (glycosylation) or signal transduction (phosphorylation). Information of how regulation is reflected in biochemical pathways can further be elucidated by metabonomics measuring endogenous metabolites. Metabonomics on biofluids such as urine and plasma is attractive due to the easy accessibility of sample material by non-invasive methods.

Integration of data between different 'omics' techniques is still at an early stage and presents a challenge for researchers working in this area, due to the large amount of data generated by these novel techniques and the absence of dedicated software tools.

The present paper has investigated the effect of hydrazine at the tiers of genes, proteins and metabolites. Hydrazine is found in diverse applications such as chemical intermediates in industry, herbicides, and rocket and aircraft fuel. In the pharmaceutical industry, hydrazine has been used in cancer therapy to counteract cachexia in terminal ill cancer patients (Gold 1987). In addition, it is a drug metabolite of two hydrazine derivatives, the antihypertensive drug hydralazine (Blair *et al.* 1985) and the antituberculosis drug isoniazid (Barlow *et al.* 1974, Noda *et al.* 1983). At high doses, hydrazine is acutely toxic causing liver damage and severe disturbances in the central nervous system (Amenta and Johnston 1962, Cornish 1969, Moloney and Prough 1983). Furthermore, it is teratogenic (Toth 1993), mutagenic (Kimball 1977) and can upon repeated administration of toxic doses produce tumours in various organs (Biancifiori 1970, Toth 1994). Following sublethal doses hydrazine causes depletion of glycogen in the liver (Fortney 1966) accompanied by dose-dependent accumulation of fat (Amenta and Johnston 1962, Timbrell *et al.* 1982, Jenner and Timbrell 1994, Waterfield *et al.* 1997b). Hydrazine has been reported to cause a number of biochemical perturbations (Moloney and Prough 1983) such as inhibiting protein synthesis (Lopez-Mendoza and Villa-Treviño 1971, Ghatineh and Timbrell 1994, Waterfield *et al.* 1997b), gluconeogenesis (Fortney *et al.* 1967, Ray *et al.* 1970) and pyridoxal-dependent enzyme reactions like transaminations (McCormick and Snell 1961, Medina 1963, Cornish 1969, Roberge *et al.* 1971, Stein *et al.* 1971). Histological examination of damaged liver tissue demonstrates steatosis with midzonal lipid vacuolation in hepatocytes with varying degrees of focal necrosis and inflammation (Scales and Timbrell 1982, Nicholls *et al.* 2001).

Using 'omics' technologies, the goal was to identify transcripts, proteins and endogenous metabolites changed by hydrazine exposure to generate novel insight into the mechanisms underlying hydrazine-induced toxicity.

Materials and methods

Chemicals and reagents

Hydrazine dihydrochloride (99.999%) and α-cyano-4-hydroxycinnamic acid were obtained from Aldrich (Milwaukee, WI, USA). CyDye^{TM} (Cy2, Cy3, Cy5) and immobilized pH gradient (IPG) strips were from Amersham Biosciences (Uppsala, Sweden). Sequencing grade, modified porcine trypsin was from Promega (Madison, WI, USA). Trifluoroacetic acid was from Sigma (St Louis, MO, USA); formic acid (Suprapur 98–100%), acetonitrile (99.8%) and sodium chloride were from Merck (Darmstadt, Germany). D_2O (99.8 atom%D) was obtained from Dr Glaser AG (Basel, Switzerland). Ultrapure water

(Milli-Q system, Millipore, Bedford, MA, USA) was used for all experiments. Other chemicals were obtained from Amersham Biosciences unless specified otherwise.

Animals and treatment

The study was conducted in accordance with Danish legislation on the use and handling of laboratory animals (Animal Welfare Act 1993, No. 726). 6 to 7-week-old male Sprague–Dawley rats (Charles River Ltd, Uppsala, Sweden) were housed individually in metabolism cages and given a standard diet (Purina chow 5002, PMI Nutrition, St Louis, MO, USA) with free access to food and water throughout the experiment. The animal facility was operated at $21 \pm 3^{\circ}C$, a relative humidity of $55 \pm 15\%$ with a 12 h light/12 h dark cycle. Rats were daily monitored for clinical signs and body weight was measured the day before dosing, at dosing and at necropsy. Rats were randomly assigned to three treatment groups: vehicle, 30 and 90 mg kg^{-1} with 10 animals per groups. All animals received a single oral dose (gavage) of vehicle (saline) or hydrazine dihydrochloride dissolved in physiological saline, with the pH adjusted to 6.5–7.5. Dose formulations were prepared on the day of administration and doses were calculated as free base. Each treatment group was divided into two equal groups: one sacrificed 48 h post-dosing, the other sacrificed 168 h post-dosing. However, the five rats from the highest dosing group were killed prematurely *in extremis* 120 h post-dosing.

For gene and protein expression analysis, liver sections from the left lateral lobe were dissected, snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ until further processing. Liver samples collected at 48 h post-dosing were subjected to gene expression analysis, while all 30 liver samples were analysed by 2D gel electrophoresis.

Blood samples for clinical chemistry were collected 24 h after dosing and shortly before sacrifice. Serum for ^{1}H-NMR spectroscopy was collected 48 h post-dosing. Serum was separated by centrifugation (about 1300 *g*, 10 min) and stored frozen at $-80^{\circ}C$ pending analysis.

Clinical chemistry and histopathology

The following parameters were measured in blood: alanine aminotransferase (ALAT), aspartate aminotransferase, alkaline phosphatase, bilirubin, gammaglutamyltransferase, carbamide, creatinine, sodium, potassium, calcium, inorganic phosphorus, protein and albumin. The parameters were measured on a RD/Hitachi 902 chemical analyser (Roche, Basel, Switzerland) using commercial test kits provided by Roche Diagnostics (Mannheim, Germany).

Liver sections for microscopic examination were fixed in 4% neutral buffered formalin, embedded in paraffin, stained with haematoxylin-and-eosin, and evaluated under a light microscope.

Gene expression analysis

To determine the relative gene expression profiles between treated and control animals, the relative abundance of mRNA was investigated using cDNA microarrays containing targets of 699 rat-specific genes selected to represent an array of genes generally involved in toxic responses observed in rats (Phase-1 Rat CT array, Ghent, Belgium). The gene expression analysis was performed at the facilities of Phase-1 Bioresearch N.V. (Ghent, Belgium). Briefly, total RNA was extracted from liver homogenates of three animals per treatment group (48 h) (four from controls) and transcribed into cDNA using reverse transcriptase in presence of dye-conjugated nucleotides. RNA from hydrazine-treated animals was individually labelled by incorporating Cy3-labelled dCTP; while RNA from control animals was pooled before addition of Cy5-labelled dCTP. After hybridization, arrays were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA) operated at wavelengths of 530 and 635 nm (excitation) and 590 and 680 nm (emission) for Cy3 and Cy5, respectively.

A twofold change in differential gene expression between treated and control rats was set as suitable threshold. Induced and reduced transcripts were coded by positive- and negative-fold changes. For each dose group, an average fold change was calculated using data from three animals.

Two-dimensional gel electrophoresis

Rat liver samples were homogenized on ice in lysis buffer (6 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, pH 8.5) by a glass homogenizer and the protein solution was centrifuged at 20 000*g* for 5 min to remove nuclei and insoluble material. The protein content of the supernatant was determined by the Bradford method (BioRad, Hercules, CA, USA) using bovine serum albumin as standard protein (Bradford 1976).

2D gel electrophoresis was performed as described in detail by Klenø *et al.* (2004b) for each of the 30 individual rat liver samples using 2D differential gel electrophoresis (2D DIGE) (Ünlü *et al.* 1997, Ünlü 1999).

In brief, the 30 liver samples were randomly assigned to either Cy3 or Cy5 labelling. The random use of Cy3 and Cy5 was done to exclude potential dye-specific variation in labelling efficiency or

fluorescence. In addition, an internal standard consisting of a pool of all 30 liver samples was labelled with Cy2, and run on each gel in the experiment. Labelling was performed by adding Cy2, Cy3 or Cy5 at a final concentration of 400 pmol fluorochrome per 50 μg protein.

Isoelectric focusing was performed in sample cups at the alkaline end, on 18-cm IPG strips (pH 4-7L). The IPG strips were loaded with a mixture of three liver protein samples consisting of two individual rat samples (Cy3, Cy5) and the internal standard (Cy2). The protein load of the individual liver samples was 100 μg total protein (total 300 μg protein per IPG strip). Separation in the second dimension was performed on 12% homogenous polyacrylamide gel electrophoresis slab gels (180 $\times$ 180 $\times$1.0 mm).

For excision of protein spots, a preparative gel was run in parallel with a total protein load of 500 μg rat liver protein. The gel was fixated for 12 h in 10% (v/v) methanol containing 7% (v/v) acetic acid and post-stained with SyproRuby (Molecular Probes, Eugene, OR, USA).

Image acquisition, relative quantification and statistical analysis of 2D DIGE gels were done using the DeCyder software from Amersham Biosciences as described (Klenø *et al.* 2004b).

Peptide mass fingerprinting by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry

For protein identification, gel spots (1.4 mm diameter) were excised using an automated spot-picker (Ettan, Amersham Pharmacia Biotech, Uppsala, Sweden), and transferred to a MALDI probe with prestructured sample support (AnchorChip™ 600 μm; Bruker Daltonik GmbH, Bremen, Germany). In-gel digestion with trypsin was performed directly on the MALDI probe (Stensballe and Jensen 2001, Klenø *et al.* 2004a).

MALDI mass spectra were acquired on an AutoFlex mass spectrometer (Bruker Daltonik GmbH). Peptide mass maps were matched against the Mass Spectrometry protein sequence DataBase (MSDB) using Mascot as search engine. Mascot settings were: no restrictions on taxonomy, p*I* or protein molecular weight, 100 ppm mass error tolerance, one missed trypsin cleavage site, cysteine residues alkylated with iodoacetamide as a fixed modification and methionine oxidation as a variable modification.

^{1}H-NMR spectroscopic analysis of serum samples

The serum samples were made up from 100 μl serum and 350 μl saline (0.9% NaCl). A total of 50 μl of a solution of formic acid in D_2O (27.9 mM) was added to provide a deuterium lock signal for the NMR spectrometer with formic acid as a chemical shift reference. ^{1}H-NMR spectra were measured at 600.13 MHz on a Bruker DRX-600 spectrometer at ambient probe temperature (298 K) using a flow-injection system (Bruker Biospin GmbH, Reinstetten, Germany). Single dimensional spectra were acquired using a standard presaturation pulse sequence for water suppression, NOESYPRESAT (Nicholson *et al.* 1995), with irradiation at the water frequency during the relaxation delay of 3 s and a pulse sequence mixing time of 100 ms. For each sample, 128 free induction decay were collected into 32 K data points with a spectral width of 12019.230 Hz and an acquisition time of about 1.36 s. Each free induction decay was multiplied by an exponential function corresponding to a 0.3 Hz line broadening before Fourier transformation.

Preprocessing of serum NMR spectra and principal component analysis (PCA)

The NMR spectra were phase corrected, baseline corrected and referenced to formic acid (δ 8.46 ppm) using an in-house routine written in MATLAB (The MathWorks, Inc., Natick, MA, USA). The same MATLAB routine also reduced each spectrum to 718 variables by integrating the spectral region δ 0.2 −7.98 into regions of 0.01 ppm width. The region δ 4.5–5.1 ppm surrounding the water resonance was excluded before spectral integration to remove the effects of variation in the presaturation of the water resonance. The reduced spectral data were then imported into the SIMCA-P+ software package (v.10.0.2.0, Umetrics AB, Umeå, Sweden). The data set consisted of four to five animals from three dose groups (vehicle, 30 and 90 mg kg^{-1}) at 48 h post-dosing. They formed a matrix (13 $\times$718) of 718 reduced NMR variables of 0.01 ppm width. All data were mean centred before PCA by subtracting from each column its average, so that all results were interpretable in terms of variation round the mean. Pareto scaled data were also used in conjunction with PCA to look for changes in less dominating metabolites in the NMR spectra. Pareto scaling weighs down the dominating variables by applying a weight of $1/\sqrt{\delta}$, where δ is the standard deviation of the variable. PCA (Wold *et al.* 1987, Martens and Næs 1993) is a projection method that reduces the dimensionality of many variables to allow representations in few dimensions where patterns of clusters or trends in the data can easily be visualized. In PCA, linear combinations of the original variables, the principal components (PCs), are calculated so that the first PC explains the largest amount of variance in the data with subsequent PCs explaining progressively less variance, all calculated PCs being orthogonal to each other. For each PC, a loading (or weight) of the original variables is calculated common for all the samples where the scores (coefficients of the PC) specify the amount of the common loading in each sample.

Results

Clinical observations, bodyweight, clinical chemistry and pathology

Toxicity was confirmed by clinical and gross macroscopic observations, clinical chemistry and histological evaluation of liver tissue(s). Animals dosed with 90 mg kg^{-1} showed from day 3 post-dosing pinched abdomen and piloerection. Furthermore, they were subdued, failed to eat and at day 5 post-dosing, they were emaciated. In line with this observation, there was a significant difference in body weight between the control and the 90 mg kg^{-1} group already 48 h post-dosing: body weight: 239 ± 10 g (control), 235 ± 6 g (30 mg kg^{-1}), 199 ± 12 g** (90 mg kg^{-1}) (**$p < 0.01$, *$p < 0.05$). These significant differences to control were calculated by one-way analysis of variance. Significant reduced activity of ALAT were observed in all hydrazine-treated animals as compared with controls: ALAT: 74.5 ± 9.2 IU ml^{-1} (control), 18.9 ± 3.7 IU ml^{-1} (30 mg kg^{-1})**, 13.7 ± 5.9 IU ml^{-1} (90 mg kg^{-1})** (48 h post-dosing). Bilirubin levels were elevated in a dose-dependent manner in treated compared with control rats indicating reduced liver function (data not shown). There was a dose-dependent reduction of both total serum protein and of albumin. Likewise, there was a dose-dependent decrease in serum creatinine. We consider that the dose-dependent reduction in protein and creatinine are at least partially, a direct effect of the marked reduction in body weight.

At necropsy at 48 h post-dosing, pale, discoloured and irregularly shaped livers were observed in the high dose animals. At histology, these livers were characterized by minimal to moderate perilobular cytoplasmatic vacuolization indicating fat accumulation. In addition, there was a higher frequency of single cell necrosis (data not shown). At 120 h (90 mg kg^{-1}) and 168 h (30 mg kg^{-1}) post-dosing, the histological evaluation revealed a more diffuse or multifocal cytoplasmatic vacuolization.

Gene expression analysis

Using cDNA microarrays, we determined the transcript levels of 699 different genes in rat liver treated with hydrazine relative to the expression in vehicle-treated animals at 48 h post-dosing. In rats treated with 30 mg kg^{-1}, 26 transcripts were induced and five were reduced at least twofold, in all treated animals (3/3) relative to controls, whereas in rats treated with 90 mg kg^{-1}, 62 and 95 transcripts were more than twofold up- or down-regulated (3/3), respectively. As such, the number of affected transcripts increased with dose reflecting a dose-related toxicity. All affected transcripts in both dosing groups exhibited similar differential expression patterns of either consistent up- or down-regulation (table 1).

Hydrazine caused regulation of mRNA levels belonging to a multitude of functional categories such as acute phase response, inflammation, immune function, stress/damage, lipid metabolism and transport, glucose regulation and biotransformation. A selection of regulated transcripts considered to be potentially involved in the pathogenesis of hydrazine hepatotoxicity is listed in table 1. A complete list of fold up- and down-regulation of mRNA levels for all hydrazine-

Table 1. Examples of liver transcripts strongly regulated 48 h after administration of a single oral dose of hydrazine.

Name[a]	AC#	Fold changes: Low dose	Fold changes: High dose	Function
Catalase	M11670	−2.6	−4.5	ROS scavenging
Gluthathione *S*-transferase α subunit/Ya	NM_031509	–	−4.5	ROS scavenging
Gluthathione *S*-transferase mu-2/Yb	NM_177426	(−2.1§§)	−5.0	ROS scavenging
Gluthathione *S*-transferase Ya	K00136	–	−3.2	ROS scavenging
Superoxide dismutase (Cu-Zn)	M21060	–	−2.2	ROS scavenging
Glutathione peroxidase	M21210	–	(−2.0§§)	ROS scavenging
Glucokinase	J04218	–	−2.5	glucose metabolism
Glucose transporter 1 (GLUT-1)	M13979	–	3.5§	glucose metabolism
Glucose-regulated protein p78	M14050	–	4.7	glucose metabolism/stress
Insulin-like growth factor binding protein 6 (IGFBP-6)	AI059172	3.2	3.0§	glucose metabolism
Apolipoprotein A-II	M28615	–	−4.2	lipid transport
Apolipoprotein C-I	AA955662	(−2.0§§)	−7.6	lipid transport
Apolipoprotein C-III	J02596	–	−2.4	lipid transport
Apolipoprotein E	J02582	–	(−2.2§§)	lipid transport
Cholesterol esterase	L46791	–	−4.4	lipid metabolism
Hepatic lipase	M16235	–	−2.6	lipid metabolism
Leptin receptor (fatty)	AA998983	2.7§	2.4§	lipid transport
Liver fatty acid binding protein	V01235	–	−7.2	lipid transport
α-Methyl-acyl-CoA-racemase		–	−4.2	lipid transport
Peroxisomal assembly factor 2	D63673	–	−6.2	lipid metabolism
Peroxisome proliferator activated receptor alpha	M88592	2.3§	2.1§	lipid metabolism
Peroxisome proliferator activated receptor gamma	AB011365	–	−3.7§	lipid metabolism
Stearoyl-CoA desaturase	AI043833	−3.3	−5.8	lipid metabolism
Sterol carrier protein 2	M34728	–	−2.7	lipid metabolism
Cytochrome P4501A1	X00469	6.6	4.5	detoxification
Cytochrome P450(2B1/2B2)	M19972	5.1	2.2§	detoxification
Cytochrome P4502C39	AA818043	–	−3.5	detoxification
Cytochrome P4502D18	AA997886	–	−3.1	detoxification
Cytochrome P45017A	M22204	2.5§	5.1	detoxification
Cytochrome P450Md	AI058887	–	−2.8	detoxification
Paraoxonase 1	U94856	–	−3.8	antioxidation
Vacuolar H^+-ATPase subunit D	AW252148	2.2§	2.9	respiratory chain/oxidative phosphorylation
ATPase inhibitor (mitochondrial IF1 protein)	AA819464	(−2.5§§)	−8.5	respiratory chain/oxidative phosphorylation

Fold-change ratio is an average based on gene expression in livers of three individual rats.

§In one of three livers, the gene expression was below twofold induction or reduction.

§§In two of three livers, the gene expression was below twofold induction or repression (data should be interpreted with caution).

[a]Gene identification is given according to GenBank.

ROS, reactive oxygen species.

treated rats can upon request be obtained from the corresponding author (Microsoft Excel file).

Protein expression analysis

The 30 male Sprague–Dawley rats were assigned to two control and four treatment groups depending on the time of necropsy at 48 or 120/168 h post-

dosing, using five animals per group. Significance levels of induction or reduction of protein expression were assessed by a Student's t-test ($p < 0.05$) between the five animals of one dose group and the corresponding group of vehicle-treated rats. Based on experimental experience with the DIGE system, only proteins that were minimum 1.3-fold induced or reduced were included.

Sixty rat liver proteins were identified by enzymatic in-gel digestion and mass spectrometry. Of these proteins, 18 showed a significant up-regulation and 18 were significantly reduced after hydrazine treatment (table 2). Of these 36 proteins, 26 were unique proteins, corresponding to 12 down- and 14 up-regulated proteins (table 2). In cases where a protein was identified in replicates, expression levels for all protein spots of the particular protein on the 2D gel are listed in table 2. Replicate protein spots of the same protein are often observed in 2D separations of eukaryotic cells and tissues, as 'trains' of spots that differ in p*I* and/or molecular mass (figure 1). These are usually isoforms of the same protein and result from a variety of post-translational modifications, e.g. phosphorylations or glycosylations.

Among the proteins that were reduced after hydrazine treatment were proteins correlated to lipid transport and metabolism. Induced proteins were related to stress response, gluconeogenesis, metabolism and transport.

Correlation between genomics and proteomics data

To determine the correlation between genomics and proteomics data, the expression levels obtained by the two techniques were compared. Fifteen of the 60 identified proteins had a counterpart on the microarray, and for 13 of these targets, there was perfect qualitatively correspondence between the protein and transcript data. However, quantitatively a general trend observed was that the transcripts were at least a factor 2 or more up- or down-regulated than the corresponding protein (figure 2). Four of these protein/transcript pairs showed unchanged expression levels between controls and treated rats both at protein and mRNA level. In one case (superoxide dismutase), the regulation of the protein was below the normal threshold of 1.3-fold regulation, but it nevertheless showed statistical significance. The two targets that showed inconsistent regulation at protein level compared with transcript level were transitional endoplasmic reticulum (TER) ATPase and catechol *O*-methyltransferase. TER ATPase was up-regulated in all dose groups at the protein level, while no regulation was observed at the transcript level (figure 2). Catechol *O*-methyltransferase showed induction at the mRNA level and no regulation of the protein level in the low dose group (48 h), while it was reduced in the high dose group (48 h) at both protein and mRNA level (figure 2). However, this apparently contradicting observation for catechol *O*-methyltransferase can be ascribed to the fewer number of animals (three) used in the gene expression analysis when compared with the protein expression analysis (five) and the large variation in the abundance of this protein in the individual rats in the low dose group (48 h) (figure 3). As such, the inconsistent result between protein and transcript of catechol *O*-methyltransferase is an artefact of our experimental set-up and not related to biology.

Table 2. Rat liver proteins showing significant differences in expression level between controls and treated animals.

Protein name[a]	Accession number, Swiss Prot.	Fold change				Functional category
		Low dose[b]		High dose[b]		
		48 h	168 h	48 h	120 h	
Alpha-2μ-globulin[a]	P02761	–	–	−2.0**	−2.7**	lipid transport
		−1.7*	–	−3.6***	−7.3***	
Apolipoprotein A-IV precursor	P02651	–	–	−1.6**	−2.0***	lipid transport
Apolipoprotein E precursor[a]	P02650	–	–	−1.3*	–	lipid transport
Carboxylesterase precursor	O35535	1.5**	–	1.5*	1.4*	metabolism
Catechol *O*-methyltransferase[a]	P22734	–	–	−1.4**	–	metabolism
Contrapsin-like protease inhibitor 6	P09006	1.5*	–	–	1.9**	inflammation
Cytokeratin 8 — Rattus norvegicus	Q10758	1.5*	–	–	1.9**	cytoskeleton
D-dopachrome tautomerase[a]	P80254	–	–	–	−1.9***	metabolism
Formyltetrahydrofolate dehydrogenase	P28037	–	–	−1.4*	−1.5**	metabolism
		–	–	−1.5**	−1.8**	
Fructose 1,6-biphosphatase	P19112	–	–	–	1.5**	gluconeogenesis
		–	–	–	1.6**	
		2.6*	2.2**	1.4**	1.9**	
Glucose-regulated protein 75	P48721	1.4***	–	1.7***	1.6**	stress
		1.2**	–	1.5**	1.3**	
Glucose-regulated protein 78[a]	P06761	–	–	1.7**	–	stress
		–	1.3*	1.7**	1.8*	
Hsc73	P08109	1.2*	–	1.3**	1.3**	stress
Hsp60	P19226	1.3*	–	1.9***	1.4*	stress
Hypothetical 79.7 kDa protein (unknown) — *Mus musculus*	Q91VD9	1.3*	–	1.3*	1.8***	other
		–	–	–	1.4***	
Kallikrein-binding protein precursor[a]	P05545	–	–	−1.9**	−2.8***	inflammation
Keratin, type I cytoskeletal 18	P05784	1.6***	1.2*	2.1***	1.3**	cytoskeleton
N-hydroxy acrylamin sulfotransferase[a]	P50237	−1.2**	–	−1.5***	−2.4***	metabolism
Regucalcin[a]	Q925W3	−1.4*	–	−1.5***	−1.9***	regulator of enzyme activity
		–	–	–	−2.0**	
		−1.5***	–	−1.4***	−2.4***	
		−1.5**	–	−1.5**	−2.0***	
Retinol-binding protein I, cellular	P02696	–	1.4*	–	1.5**	transport

Table 2 (*Continued*)

Protein name[a]	Accession number, Swiss Prot.	Fold change: Low dose[b] 48 h	Low dose[b] 168 h	High dose[b] 48 h	High dose[b] 120 h	Functional category
Serum paraoxonase[a]	P55159	–	– 1.2**	−1.3*	−2.3**	antioxidation
Superoxide dismutase (Cu-Zn)[a]	P07632	–	–	–	−1.2*	ROS scavenging
Transitional endoplasmatic reticulum ATPase[a]	P46462	2.0***	1.8**	2.7***	2.0**	transport
Translation initiation factor eIF-4A I — mouse	P04765	–	–	1.5**	1.4*	transcription
Transthyretin[a]	P02767	–	–	−2.2***	−1.7**	transport
Tropomyosin 5, fibroblast	P97726	1.2*	–	1.5***	1.4**	cytoskeleton
Tubulin alpha chain	P02551	–	–	–	−2.5**	cytoskeleton

[a]Proteins with counterparts on the employed microarray.

[b]Negative-fold change ratio different from 1 indicates that the protein was reduced in the group administrated hydrazine compared with the group receiving vehicle, while a positive value corresponds to an induction of the protein in the hydrazine-treated animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ROS, reactive oxygen species.

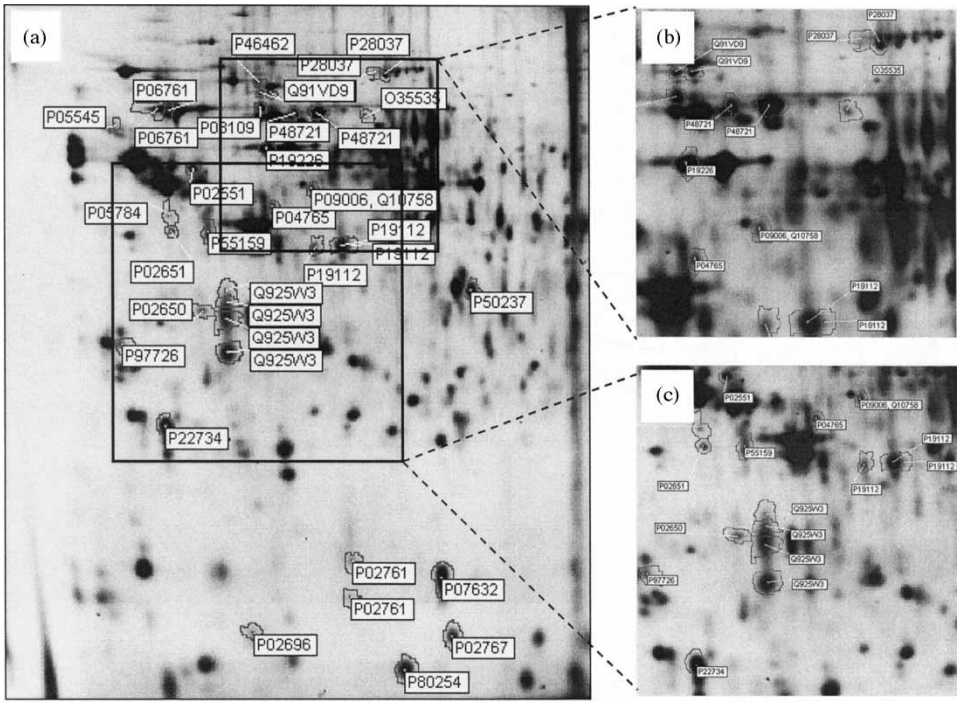

Figure 1. (a) Cy3-labelled two-dimensional gel separation of rat liver proteins. Isoelectric focusing was performed on an immobilized pH 4–7 gradient followed by separation in the second dimension on 12% polyacrylamide gels. Rat liver proteins with changed expression levels after hydrazine treatment are annotated with the Swiss Prot. accession number. (b) Horizontal lanes of four proteins corresponding to P28037, Q91VD, P48721 and P19112. (c) Enlargement of area of two-dimensional gel containing a vertical lane of regucalcin (Q925W3).

Metabolite profiling using NMR and PCA

Figure 4 shows representative ^{1}H-NMR spectra of a serum sample from a rat treated with 90 mg kg^{-1} hydrazine (A) and a vehicle serum sample (B) collected at 48 h post-dosing. A comparison of the two spectra shows some distinct differences between the spectral profiles. Most of the broader peaks have clearly decreased in the hydrazine-treated spectrum (A) when compared with the control spectrum (B). These broad peaks are assigned to lipids and lipoproteins in the serum samples. Other metabolites such as citrulline and 2-amino adipic acid appeared in treated (A) but not in control rats (B) and represent metabolites that are related to the hydrazine treatment. The metabolites indicated in the spectra in figure 4 were assigned using previous studies of rat serum samples (Nicholson *et al.* 1995, Nicholls *et al.* 2001).

Having inspected the NMR spectra to get a preview of the spectral changes caused by hydrazine treatment, PCA was applied to the NMR data to find the general pattern of dose-related changes in all metabolites in the spectra at 48 h post-dosing. Figure 5 shows the PCA score plot of the first principal component (PC1) versus the second principal component (PC2) of mean centred data. The first two components represent 91% of the total variance in the NMR serum data.

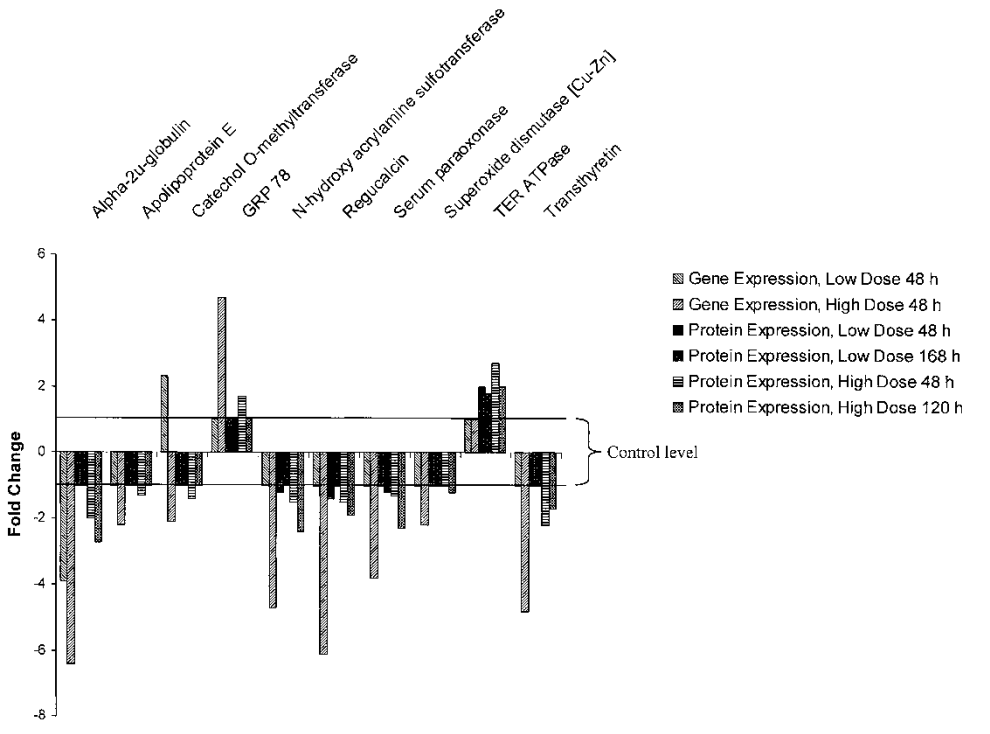

Figure 2. Comparison of gene and protein expression levels after treatment with a high (90 mg kg^{-1}) and low (30 mg kg^{-1}) dose of hydrazine. The average for each gene is calculated from three rats, while the average for each protein is calculated from five rats.

The plot shows a good separation of the three clusters of animals from the control group (◆), animals that have been given 30 mg kg^{-1} hydrazine (■) and animals that have received a dose of 90 mg kg^{-1} hydrazine (▲). The PCA model of Pareto scaled data (data not shown) also displayed a good separation of the three clusters in the first two PCs. Corresponding loading plots (data not shown) of the mean centred and Pareto scaled PCA models were used to find the patterns of metabolites that contributed to the dose-related separation in the models. These metabolites are listed in table 3 and their positions are indicated in the NMR spectra in figure 4. Decreased levels of lipoproteins and lipids in hydrazine-treated animals contributed to the dose-related separation in the PCA models together with decreased levels of glucose and choline. Dose increased levels of amino acids such as alanine, valine and tyrosine also contributed to the cluster separation in the models. Citrulline and 2-amino adipic acid strongly contributed to the models because the two metabolites only appeared in the NMR spectra at 90 mg kg^{-1} dose.

Discussion

Previously published data have shown a maximal hepatotoxic response at 40–60 mg kg^{-1} (Scales and Timbrell 1982, Timbrell *et al.* 1982, Preece *et al.* 1992). Therefore, we chose to treat the rats with flanking doses at 30 and 90 mg kg^{-1}.

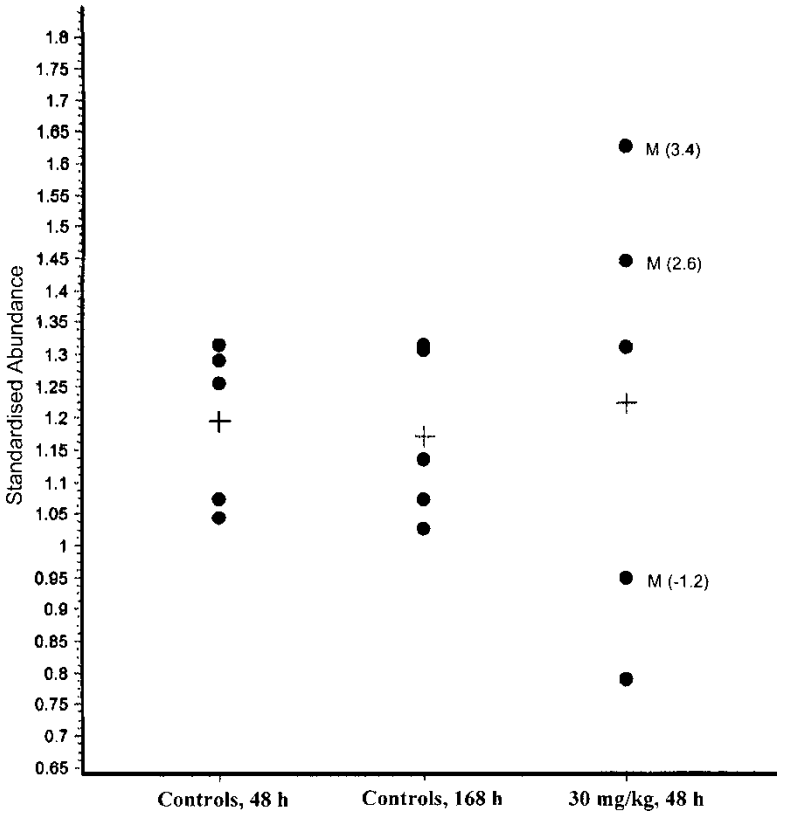

Figure 3. To show that mRNA and protein expression of catechol *O*-methyltransferase correlate, the values for the individual rats are shown in the figure. Each circle represents the protein abundance in an individual rat. The 'plus' signs indicate the average of protein expression for each group. The three animals in the low-dose group (48 h) that also were analysed by microarray are annotated with an M. The fold changes for mRNA are noted in parentheses.

Based on the clinical observation and the macroscopic, histopathological and clinical chemistry findings in rats treated with 90 mg kg^{-1}, a toxic injury consistent with the observations made by others (Scales and Timbrell 1982, Timbrell and Waterfield 1996, Nicholls *et al.* 2001) was observed.

To gain insight into the molecular mechanisms of hydrazine toxicity, we compared the hepatic gene and protein expression profiles between hydrazine-treated and control rats. Information of how regulation was reflected in biochemical pathways was further elucidated by metabonomics measuring endogenous metabolites by ^{1}H-NMR. An excellent agreement between the expression results of microarrays and 2D gel electrophoresis was observed, supporting the expectation that a decreased or increased transcription of a gene should result in a repression or induction of the corresponding protein. Only two of 15 transcript/protein pairs showed inconsistency in regulation (figure 2). TER ATPase showed up-regulation in all dose groups at the protein level, while no regulation was observed on the transcript (figure 2). This inconsistency for TER ATPase could potentially be explained by differences in half-life for the corresponding mRNA and protein so that the transcript may have been up-regulated at an earlier time point not

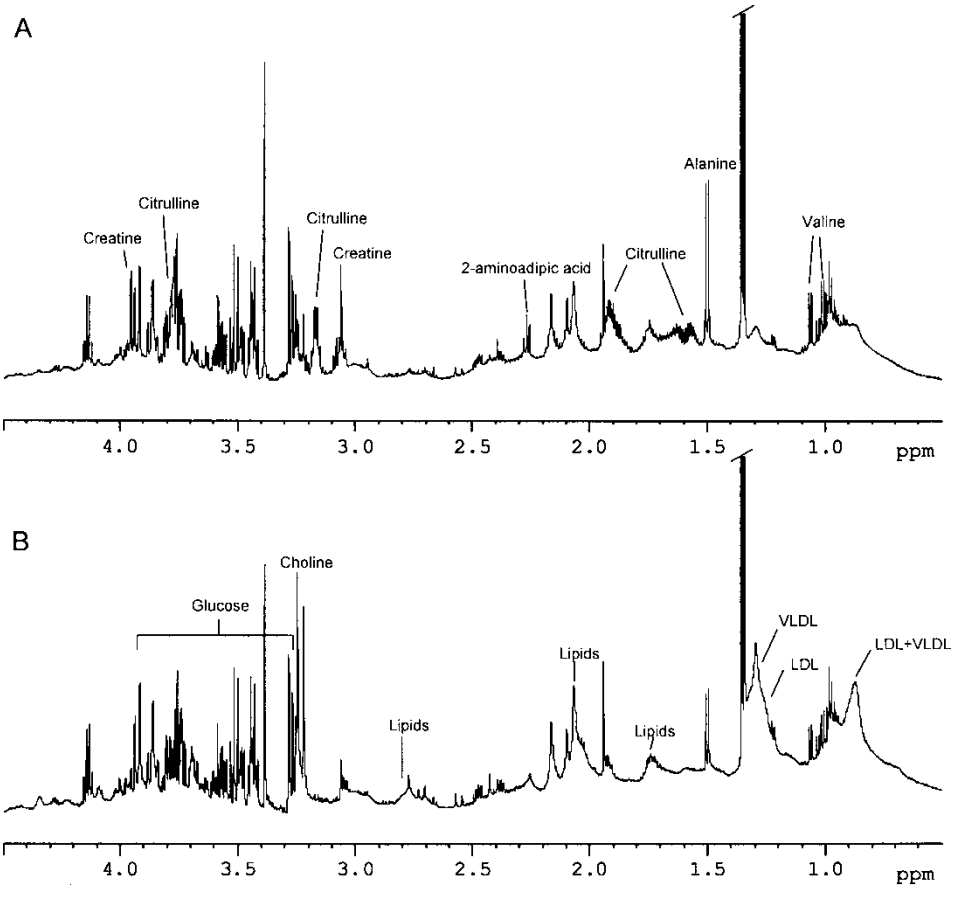

Figure 4. Partial ^{1}H-NMR spectra (δ 0.5–4.5) with water presaturation of whole rat serum at 48 h post-dosing from an animal administered hydrazine 90 mg kg^{-1} (A) and a control (B). The metabolites that visibly have changed with hydrazine treatment are indicated in the spectra. VLDL, very low density lipoproteins; LDL, low density lipoproteins.

investigated in the current study. Catechol *O*-methyltransferase showed induction at the mRNA level and no regulation of the protein level in the low dose group (48 h), while it was reduced in the high dose group (48 h) at both protein and mRNA level (figure 2). This apparently contradicting observation for the low dose group can, however, be ascribed to the large variation in the abundance of this protein in the individual rats in the low dose group (48 h) (figure 3). Protein expression analysis was performed on all five rats included in this group, whereas gene expression analysis was performed on only three animals that coincidentally had a higher level, on average (figure 3). This stresses the importance of including enough animals in toxicity studies to avoid the misinterpreting of results. Interestingly, a general trend observed in the current study was that the transcripts were at least a factor 2 or more affected than the corresponding protein. According to the literature, hydrazine causes a general inhibition of the protein synthesis (Lopez-Mendoza and Villa-Treviño 1971, Ghatineh and Timbrell 1994, Waterfield *et al.* 1997b). This could at least explain why we observe less up regulation of proteins

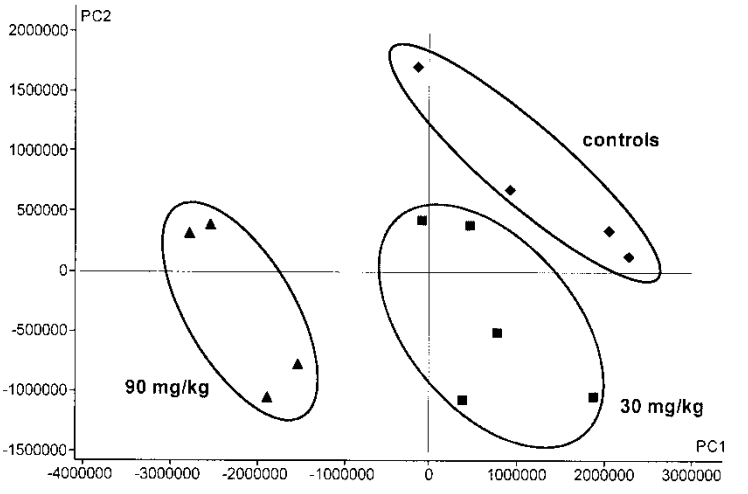

Figure 5. Score plot of principal component 1 (PC1) versus principal component 2 (PC2) from a principal component analysis model of mean centred nuclear magnetic resonance data in 0.01 ppm segments from serum of controls and hydrazine-treated rats at 48 h post-dosing. Controls (♦), 30 mg kg^{-1} (■), 90 mg kg^{-1} (▲). The first two principal components explained 91% of the total variance in the spectral data.

when compared with their corresponding mRNAs. When it comes to the observed down regulations it might be that the generally short half lives for mRNAs when compared with proteins can be the explanation for our observation.

Several of the transcripts, proteins and metabolites identified as regulated in this study are related to perturbations of gluconeogenesis, lipid synthesis and transport, oxidative stress and mitochondrial function (tables 1–3).

Lipid transport and metabolism

One of the major affects of hydrazine is accumulation of fat in the liver causing liver steatosis.

Table 3. Assignments of endogenous metabolites that contributed highly to the hydrazine dose-related separation in the principal component analysis models of nuclear magnetic resonance spectra of serum samples at 48 h post-dosing.

Metabolites[a]	^{1}H chemical shifts (δ) (ppm)[b]	Increase (↑) or decrease (↓) after hydrazine exposure
Choline	3.21	↓
Glucose	3.25, 3.42, 3.46, 3.48, 3.54, 3.83, 3.9	↓
Lipoproteins (VLDL, LDL)	0.85–0.88, 1.2–1.3	↓
Lipids	1.7, 2.05, 2.78	↓
Alanine	1.48	↑
2-Amino adipic acid	2.23	↑
Citrulline	1.57, 1.88, 3.15, 3.75	↑
Creatine	3.04, 3.93	↑
Tyrosine	6.9, 7.2	↑
Valine	0.99, 1.04	↑

[a]Assigned from literature values (Nicholson *et al.* 1995, Nicholls *et al.* 2002).
[b]Chemical shifts for identification of metabolites as indicated in figure 4, except tyrosine, which peaks were outside the displayed spectral region.

The pathogenesis underlying liver steatosis is still not fully elucidated. Mechanisms that synergistically have been proposed to contribute to fat accumulation in hepatocytes include: increased influx of fatty acids, increased synthesis of lipids, reduced utilization of lipids by decreased β-oxidation and decreased efflux of lipids from hepatocytes to the circulation (Amenta and Dominguez 1965, Waterfield *et al.* 1997a).

All three 'omics' techniques concurrently demonstrated that hydrazine at toxic doses disturbs lipid transport from the liver, and the data suggest that both the production of lipoproteins and β-oxidation of fatty acids are impaired following hydrazine treatment. In serum, lipoproteins (very low density lipoproteins [VLDL], low density lipoproteins [LDL]), lipids, and choline were clearly decreased in hydrazine-treated rats (figure 4 and table 3) as an indication of decreased transport of lipids from the liver. Choline deficiency has previously been associated with liver steatosis in rats (Kaminski *et al.* 1980, Buchman *et al.* 1992). In addition, several proteins and transcripts associated to lipid metabolism and transport were significantly regulated (tables 1 and 2).

Lipids synthesized in the liver are solubilized in lipoprotein complexes that consist of an apolar core and a polar shell with apoproteins floating in the membrane. Simplistically, VLDL transports triacylglycerol, cholesterols, cholesteryl esters and phospholipids to extrahepatic tissue. Lipoprotein lipase (LPL), which is present in endothelial cells in fat and muscle capillaries, catalyses the uptake of fatty acids. Apolipoprotein (Apo) C-II and insulin in adipose tissues activate LPL (Andersson *et al.* 1999, Pedersen *et al.* 1999), whereas Apo C-I and C-III inhibit LPL (Shachter 2001). Since the gene expression of Apo C-I and Apo C-III was suppressed (table 1), this could lead to a higher activity of LPL and thus lower levels of circulating lipids.

Excess cholesterol from tissues returns back to the liver fixed to HDL particles. Before transport back to the liver, cholesterol is acylated to cholesteryl ester by lecithin:cholesterol acyl transferase (LCAT). Apo A-I, C-I and A-IV can activate LCAT (Soutar *et al.* 1975, Steinmetz and Utermann 1985, Daum *et al.* 1999, Shachter 2001). We saw a down-regulation of the expression of Apo C-I (gene) and Apo A-IV (protein) following hydrazine treatment, which could indicate reduced LCAT levels thereby diminishing the reverse transport of cholesterol to the liver. However, we did not see major changes in differential expression levels of transcripts for LPL and LCAT in hydrazine-treated rats, and consequently we cannot make any conclusions about the specific mechanisms of action of hydrazine on lipoprotein metabolism apart from stating that lipoprotein release to the blood is reduced and the synthesis of apolipoproteins is disturbed.

Electron microscopy analysis of hepatocytes exposed to hydrazine has revealed major ultrastructural changes as mitochondria appear swollen and fuse to form megamitochondria (Wakabayashi *et al.* 1987, Matsuhashi *et al.* 1997, Karbowski *et al.* 1997). In addition, there is an abnormal high number of peroxisomes in association to them (Scales and Timbrell 1982). Degradation of fatty acids by β-oxidation mainly takes place in the mitochondria, while very long fatty acids are oxidized in peroxisomes before they proceed to final degradation in mitochondria (Reddy 2001). In hydrazine-treated rats we observed suppressed transcript

expression for peroxisomal assembly factor 2 involved in biogenesis of peroxisomes (Zhang *et al.* 1999) and for α-methyl-acyl-CoA racemase (table 1). The racemase is a peroxisomal auxiliary enzyme involved in a stereoselective conversion of (2R)-pristanoyl-CoA and (25R)-di and tri-hydroxycholestanoic acid (bile acid intermediates) to their *S*-forms before further degradation by peroxisomal β-oxidation takes place (McLean *et al.* 2002, Cuebas *et al.* 2002). A repression of this racemase would imply impaired degradation of particularly long branched chain fatty acids (Reddy 2001, McLean *et al.* 2002, Cuebas *et al.* 2002).

Furthermore, we saw the gene expression of peroxisome proliferator activated receptor (PPAR)α, an isoform expressed primarily in tissues with high catabolism of fatty acids such as liver, was induced in rats exposed to hydrazine, concomitantly PPARγ, an isoform expressed mainly in fat tissues, was reduced (Lee *et al.* 2003). The peroxisome proliferator activated receptors are a small group of nuclear hormone receptors which through their activation have a pivotal role in regulating intermediary metabolism. PPARα and PPARγ have almost oppositely directed actions. Following conditions of low blood glucose levels, PPARα becomes activated and stimulate uptake and β-oxidation of fatty acids, promote synthesis of glucose through activating genes involved in gluconeogenesis and regulate the synthesis of selected apolipoproteins. Oppositely, PPARγ activation stimulates adipogenesis and suppress gluconeogenesis by down-regulating the levels of e.g. phosphoenolpyruvate carboxykinase (PEPCK) (Kersten 2002, Lee *et al.* 2003). We propose that induction of PPARα expression and a repression of PPARγ expression could represent a physiological mechanism to counteract glucose and energy shortage caused by hydrazine.

Glucose metabolism

Three transcripts (glucokinase, glucose tranporter 1 (GLUT-1), insulin-like growth factor binding protein (IGFBP-6) (table 1), and one protein, fructose 1,6-bisphosphatase (table 2), involved in the glucose metabolism were up-regulated except for glucokinase which was down-regulated. In addition, hydrazine treatment caused up-regulation of glucose regulated proteins, Grp 75 and Grp 78 (table 2), a special group of heat shock proteins induced by, for example, low extracellular glucose concentration (Snoeckx *et al.* 2001). Toxic doses of hydrazine cause hypoglycaemia with depletion of glycogen (Fortney *et al.* 1967, Fortney 1967, Aleyassine and Lee 1971, Cooling *et al.* 1979, Moloney and Prough 1983). Hypoglycaemia was indeed reflected in the metabonomics data, where a decrease of glucose in serum was observed after hydrazine treatment (table 3). It is likely, that the liver cannot maintain normoglycemia by *de novo* synthesis of glucose because hydrazine directly interferes with the gluconeogenesis (Fortney *et al.* 1967, Ray *et al.* 1970, Moloney and Prough 1983). Hydrazine forms stable Schiff's base adducts with pyridoxal 5-phosphate (PLP) (McCormick and Snell 1961, Fortney 1967, Cornish 1969) and 2-oxoacids (Fortney 1967, Moloney and Prough 1983, Ghatineh *et al.* 1992). Gluconeogenesis is partly sustained by the catabolism of amino acids to glucose, where amino acids, most importantly aspartate and alanine, are transported to the liver to undergo transamination to form oxaloacetate and pyruvate catalysed by PLP-dependent transaminases. Forty-eight hours post-

dosing, we observed an increased level of alanine in serum in hydrazine-treated animals (table 3 and figure 4A) accompanied with reduced activity of ALAT in serum. This is likely a result of reduced function of alanine transaminase arising from the lack of available PLP due to hydrazone formation with hydrazine. Reduced function of amino transaminases may also be responsible for the elevations in amino acids we observed (table 3 and figure 4A). Hydrazines have been postulated not only to deplete oxaloacetate supplies by inhibiting transaminase activity but also through removal of oxaloacetate as a hydrazone (Fortney 1967, Kleineke *et al.* 1979, Moloney and Prough 1983). In the mitochondrion or in the cytosol, oxaloacetate is converted to phosphoenolpyruvate catalysed by the enzyme PEPCK. Hydrazine can non-competitively block PEPCK *in vitro* (Ray *et al.* 1970). This enzyme is together with fructose 1,6-bisphosphatase key regulatory enzymes selective to the gluconeogenesis (figure 6). We observed a relative overexpression of the protein level of fructose 1,6-bisphosphatase in hydrazine-treated animals. This enzyme catalyses the conversion of fructose 1,6-bisphoshate to fructose 6-phosphate before this enters the endoplasmatic reticulum for conversion to glucose (figure 6).

The glucokinase transcript, which was found to be down-regulated catalyses the conversion of glucose to glucose 6-phosphate, the first step in the glycolysis. Glucokinase is a liver specific isoform of hexokinase and function to allow the liver to buffer blood glucose. The synthesis of glucokinase is hormonally regulated (Weinhouse 1976, Minderop *et al.* 1987, Silverstein *et al.* 1989) and under conditions of low blood glucose and low insulin levels, liver glucokinase becomes

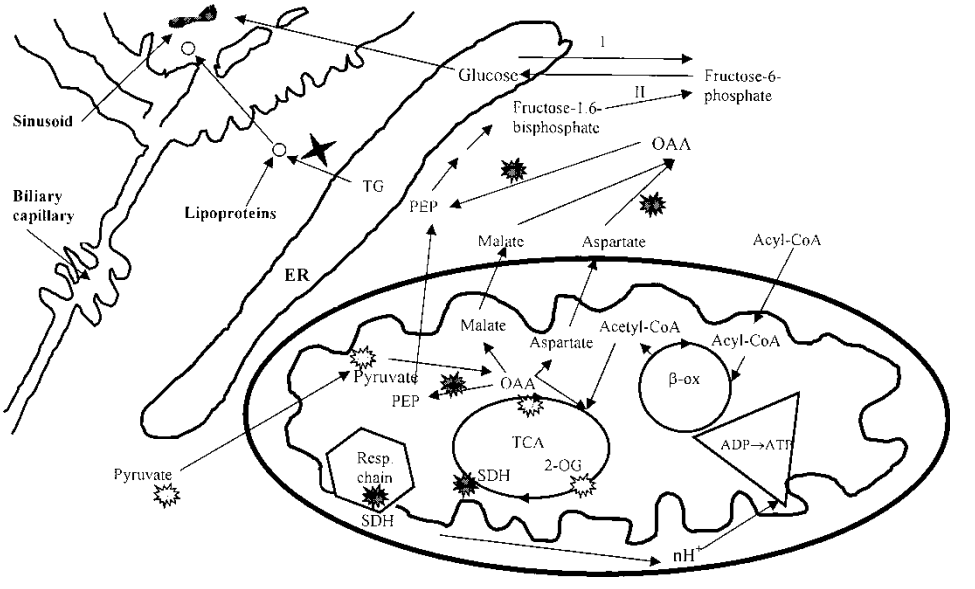

Figure 6. Possible mechanisms of toxicity of hydrazine focusing on lipid, glucose and energy metabolism. Simplified drawing of section of hepatocyte. ✸, Enzyme inhibition; ✸, hydrazone formation; ✶, reduced secretion; TG, triacylglycerols; ER, endoplasmatic reticulum; SDH, succinate dehydrogenase; 2-OG, 2-oxoglutarate; OAA, oxaloacetate; SDH, succinate dehydrogenase; PEP, phosphoenolpyruvate; I, glucokinase; II, fructose 1,6-bisphosphatase.

inactive thereby allowing scarce glucose supplies to become available for tissues like brain (figure 6). GLUT-1 is one of the isoforms of the glucose transporters, expressed in most tissues, which by its translocation to the cell surface facilitates the transport of glucose into cells (Ismail-Beigi 1993, Hwang and Ismail-Beigi 2002). Cells transfected to overexpress GLUT-1 exhibit stimulated rates of glucose transport. GLUT-1 expression has been found to be up-regulated in rat liver tissues from animals with pathologically glycogen depleted livers (Nanji *et al.* 1995).

We hypothesize that a down-regulation of the glucokinase expression, an induction of GLUT-1 mRNA transcripts and an up-regulation of fructose 1,6-bisphosphatase enzyme levels reflect a compensatory response to meet the demand of glucose to vital tissues in consequence of an attenuated gluconeogenic activity caused by hydrazine.

Oxidative stress and mitochondrial function

Evidence of lipid peroxidation and oxidative stress leading to compromised cell integrity has been demonstrated in cultured hepatocytes exposed to hydrazine and in isolated liver cells from hydrazine-treated rats (Di Luzio *et al.* 1973, Matsuhashi *et al.* 1997, Karbowski *et al.* 1997, Hussain and Frazier 2002). In the present study, six transcripts — catalase, gluthathione *S*-transferase α subunit, gluthathione *S*-transferase mu-2, gluthathione *S*-transferase Ya, superoxide dismutase (Cu-Zn) (SOD1), gluthathione peroxidase — and one protein, SOD1, involved in scavenging of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, were significantly down-regulated in hydrazine-treated animals.

Hepatocytes live in a constant balance of ROS production, scavenging and repair of damages caused by free radicals. ROS react with all classes of cellular macromolecules, and the reactions they undergo have been implicated in a variety of disease states, including atherosclerosis, stroke, cancer, emphysema and ageing. Non-enzymatic antioxidants like vitamins E and C and gluthatione (GSH) and enzymatic antioxidants such as superoxide dismutase, catalase, gluthatione peroxidase and gluthatione *S*-transferase prevent cellular damage from ROS (Klaunig *et al.* 1995). It has been reported that hydrazine at concentrations of 25 mM and higher significantly reduces catalase and GSH activities in hepatocytes compared with untreated controls paralleled by cytotoxicity (LDH leakage), decreased mitochondrial function, increased lipid peroxidation and formation of ROS (Hussain and Frazier 2002).

SOD1 is found in the cytosol of eukaryotic cells and is the first line of defence of oxidative damage among enzymatic mechanisms. This metalloenzyme catalyse a dismutation, i.e. a reaction in which two superoxide molecules undergo a redox reaction, under formation of hydrogen peroxide and molecular oxygen (Sreekumar *et al.* 2003). Hydrogen peroxide is further detoxified by catalase to oxygen and water. Catalase is a widely distributed haem protein, with a rapid turnover rate found in high concentrations in the liver. Gluthathione peroxidase, most abundant in erythrocytes, catalyses the oxidation of hydrogen peroxide into water converting GSH to oxidized gluthathione (GSSG). The regeneration of GSH is catalysed by

gluthathione reductase. GSH, gluthathione peroxidase and reductase work together and deficiency in any of those is believed to be associated with an increased sensitivity of the cell to oxidative stress (Klaunig *et al.* 1995).

Sreekumar *et al.* (2003) studied the hepatic expression profiles of 6400 known human genes in six healthy volunteers and in six patients with cirrhotic stage non-alcoholic steatohepatitis (NASH). Interestingly, they found significantly suppressed expression of transcripts involved in ROS scavenging (i.e. SOD1, catalase and aldehyde oxidase) in patients with NASH compared with controls and concluded that this could be of importance for mitochondrial function (Sreekumar *et al.* 2003). ROS are generated in mitochondria and peroxisomes mainly during breakdown of fatty acids and as by-products from the respiratory chain. Reduced expression of genes and proteins associated to ROS scavenging could contribute to radical formation attacking proteins, DNA and lipids within the mitochondria. As for NASH, several findings suggest that mitochondrial dysfunction could be an important hallmark in the pathogenesis of hydrazine (Matsuhashi *et al.* 1997, Hussain and Frazier 2002). The mitochondria contain enzymes for the citric acid cycle, β-oxidation, urea synthesis, respiratory chain and ATP synthesis (figure 6). Several studies have shown that exposure of hepatocytes to hydrazine causes ATP depletion (Preece *et al.* 1990, Ghatineh *et al.* 1992). The depletion could be linked to the observation that hydrazine at non-cytotoxic concentrations inhibits the activity of succinate dehydrogenase (SDH) (Ghatineh *et al.* 1992). In the mitochondrion, this enzyme resides in the intermembrane space, where along with other enzyme complexes it transports protons that pumped back into the matrix space is coupled to ATP synthesis. In addition, SDH is found in the matrix space catalysing the conversion of succinate to fumarate in the citric acid cycle (Ghatineh *et al.* 1992). Consequently, a inhibition of SDH would likely reduce ATP production from the mitochondrion. Two transcripts, vacuolar H^+-ATPase subunit D and an ATPase inhibitor and one protein TER ATPase associated to oxidative phosphorylation are differentially expressed in hydrazine-treated rats indicating that we see disturbances in energy synthesis.

Further, we observed a down-regulation of mRNA and protein expression for serum paraoxonase/arylesterase (PON1) following hydrazine treatment. PON1 is a high density lipoprotein (HDL)-bound protein (Durrington *et al.* 2001). Several studies suggest that the association with HDL may protect against low density lipoprotein (LDL) oxidation (Mackness *et al.* 2002) known to contribute to the onset and progression of atherosclerosis (Banka 1996, Durrington *et al.* 2001, Mackness *et al.* 2002). In certain disease states associated with oxidative stress and lipid peroxidation, reduced levels of PON1 in plasma and liver tissues has been detected (Mackness *et al.* 2002). Thus, a down-regulation of PON1 following hydrazine treatment could be viewed as a causative response to oxidative stress.

Conclusion

An excellent qualitatively agreement between the expression results of micro-arrays and 2D electrophoresis was observed. This conclusion was based on 15 targets identified both in the protein and gene expression analysis. The number of

identical protein/transcript pairs would likely increase if isoelectric focusing was performed using a broader p*I* gradient, e.g. 3–11.

It must be stressed, however, that genomics and proteomics are complementary techniques. While microarrays enable a very large number of genes to be studied, proteomics enables identification of post-translational modifications, which can alter the function of the proteins after translation. This can have biological consequences such as altered signal transduction, protein half-life, proteolysis, etc. If the protein/transcript data are joined with information of how regulation is reflected in biological pathways by measuring the level of endogenous metabolites, an extremely powerful tool to elucidate or support hypothesis of molecular mechanisms involved in toxicity is obtained.

In the present study, evidence of impairment of lipid and glucose metabolism for instance was reflected with all three techniques.

In conclusion, combination of genomic, proteomic and metabonomic data provides a unique tool to reveal molecular mechanisms underlying toxicity and enables identification of a wide range of potential biomarkers that can be monitored in clinical trials of drug development.

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