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A proteomic method for analysis of CYP450s protein expression changes in carbon tetrachloride induced male rat liver microsomes

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Abstract

Carbon tetrachloride (CCl_4) is a well-known model compound for producing chemical hepatic injury. Cytochrome P450 is an important monooxygenase in biology. We investigated the CYP450 protein expression in the *in vivo* hepatotoxicity of rats induced by CCl_4. In this experiment, CCl_4 were administered to male rats, and their livers at 24 h post-dosing were applied to the proteomic analysis. Blood biochemistry and histopathology were examined to identify specific changes. At the same time, a novel acetylation stable isotopic labeling method coupled with LTQ-FTICR mass spectrometry was applied to disclose the changes of cytochrome P450 expression amounts. The quantitative proteomics method demonstrated its correlation coefficient was 0.9998 in a 100-fold dynamic range and the average ratio of the labeled peptides was 1.04, which was very close to the theoretical ratio of 1.00 and the standard deviation (S.D.) of 0.21. With this approach, 17 cytochrome P450 proteins were identified and quantified with high confidence. Among them, the expression amount of 2C11, 3A2, and 2 E1 were down-regulated, while that of 2C6, 2B2, and 2B1 were up-regulated.

Keywords: Carbon tetrachloride; Cytochrome(s) P450; Quantitative; Proteomics; Hepatic injury

1. Introduction

Carbon tetrachloride (CCl_4), widely used to elicit experimental liver damage, is a potent environmental hepatotoxin. The hepatic damage induced by CCl_4 causes significant rise in marker enzymes ASP and ALP. The effects of CCl_4 on hepatocytes are manifested histologically as hepatic steatosis, fibrosis, hepatocellular death, and carcinogenicity (Junnila et al., 2000). Its toxic effect is believed to be due to trichloromethyl radical which is formed by an unstable metabolic intermediate (Stoyanovsky and Cederbaum, 1999). In the biotransformation process, carbon tetrachloride is catalysed by cytochrome P450 enzymes, leading to the formation of the reactive trichloromethyl radical.

The superfamily of cytochrome(s) P450 (P450) is one of the important monooxygenase system components, which plays a criteria role in the hepatic and extrahepatic drug metabolism. Most of P450 proteins are located in the membranes of liver cells as well as

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in many other tissues (e.g. lung, intestine and kidney) (Kanaeva et al., 2005).

Recently, the identification of P450 proteins was a major subject of monooxygenase system and hundreds of P450 were discovered (Alterman et al., 2005), subsequently the mechanism of some cytochrome P450s was undertaken and the interaction between P450 proteins (Casabar et al., 2006; Galetin and Houston, 2006; Gupta et al., 2005) and the action of P450 to the other substrates (Zhu et al., 2006; Turini et al., 1998) were well known increasingly. Several common techniques for the cytochrome P450s identification are enzyme activity assays, protein immunodetection and the detection of mRNA (Chen et al., 2002). Immunoblotting requires forms-specific antibodies of cytochromes P450. The detection of mRNA is not reliable enough as protein expression is not exactly related to mRNA. Enzyme activity assays also will bring uncertain results due to the cross-reactivity of marker substrates (Kanaeva et al., 2005). A prominent issue is such researches on single or partial P450 proteins could not demonstrate a comprehensive function of P450 super family in a metabolic processing of a particular drug or in examination of the potential drug–drug interaction. In addition, those low levels of multiple proteins are hard to detect by the low-throughput methods. Proteomic analyses of the P450 isozyme expression provide an alternative strategy to monitor the up- and down-regulation of all the P450 proteins so that a general interaction of P450s will be made out. In 2005, Petushkova and co-workers (Kanaeva et al., 2005) combined proteomics with biochemical method to analyze the mouse liver microsomes. Meanwhile, Patterson and co-workers (Lane et al., 2004) identified cytochrome P450 enzymes in human colorectal metastases and the surrounding liver with SDS–PAGE nano-LC-ESI-MS/MS and consequently 14 distinct p450 enzymes were revealed from the subfamilies1A, 2A, 2B, 2C, 2D, 2E, 3A, 4A, 4F, 8B and 27A. The most important of all were the distinction of P450 expression between liver samples and tumor tissue.

However, the identification method is merely able to tell us the existence or inexistence of the P450 enzyme in the tissue but can not distinct the expression changes of a single enzyme in two tissues. Consequently, quantitative proteomics strategy is introduced to the research of cytochrome P450. Jenkins et al. (2006) applied isotope-coded affinity tags (ICAT) strategy to the relative and absolute quantitative expression profiling of cytochromes P450. Based on the discriminatory power and throughput, ICAT technology superseded conventional methods for P450 profiling and simultaneous detected distinct induction profiles for CYP2C subfamily members in response to phenobarbitone.

In recent years, stable labeling technology has been applied in the quantitative proteomics research. During lots of labeling strategies, acetylation tagging aiming at the primary amine is a simple and efficient method. Fayun (Che and Fricker, 2002) had accurately quantified the difference of neuropeptides in Cpe^{fat}/Cpe^{fat} mice using d0/d6-acetic anhydride labeling strategy.

Meanwhile, FT mass spectrometry with its high resolution and accuracy became increasingly popular in quantitative analysis instrument. Gygi and co-workers (Everley et al., 2006) also considered LTQ-FT MS a good instrument for the quantitative analysis through the research of metastatic prostate cancer. The number of quantified proteins was three-fold more than the previous research results.

In this study, acetylation stable isotopic labeling technique coupled with LTQ-FTICR-MS was used for the quantitative analysis of cytochrome P450 expression profiling. The aim of our study was to adopt a proteomic analysis of P450 in CCl_4 damnifying and natural livers, and to reveal which P450 enzymes would be in response to CCl_4.

2. Materials and methods

2.1. Chemicals and reagents

Horse heart myoglobin and d6-acetic anhydride were purchased from Sigma–Aldrich (St. Louis, MO). Dithiothreitol (DTT) and sequencing grade modified trypsin were purchased from Promega (Madison, WI). Iodoacetamide (IAA), trifluoroacetic acid (TFA), tetrahydrofuran (THF) and *O*-methylisourea hemisulfate were obtained from Acros Organics (Belgium, USA). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, USA). Deionized water was produced by a Milli-Q A10 System from Millipore (Bedford, MA). Formic acid (FA) was from Fluka (USA). d0-acetic anhydride was obtained from Beijing chemical company (Beijing, China). Solid phase extraction was bought from Supelco (Bellefonte, USA). Carbon tetrachloride was purchased from Shanghai chemical reagent company (Shanghai, PR China). All other chemicals were analytical reagent grade.

2.2. Animals and treatments

The animals used in this studies were 180–220 g fed male Sprague–Dawley rats from Shanghai SLAC Laboratory Animal CO. LTD. They were housed in polypropylene cages under standard animal house conditions with a 12 h light/dark cycle at a temperature of $25 \pm 2\,^{\circ}C$. The animals had free access to food and water and were divided into two groups, with each group containing four animals. Group A (control group) received corn

oil. Group B (induction group) received CCl_4 1 ml/kg body weight in 1 ml corn oil, intraperitoneally. This dose which represents approximately one third of the LD_{50} dose in rat (Klingensmith and Mehendale, 1982) was chosen to ensure hepatocellular necrosis with low or negligible mortality.

2.3. Microsome preparation

Rats were sacrificed by decapitation. Rats' livers were quickly removed and transferred to ice-cold 0.02 M Tris–1.15% KCl, pH 7.4 buffer. Microsomal fractions were prepared essentially as described previously (Buckpitt et al., 1983). All subsequent procedures were performed on ice. Pooled livers were removed from buffer, blotted, and weighed. Homogenization was in three volumes of ice-cold buffer (0.02 M Tris–1.15% KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 15% glycerol, pH 7.4), and resultant homogenate was centrifuged at $10{,}000 \times g$ for 30 min; the supernatant was then removed and centrifuged at $105{,}000 \times g$ for 1 h. The microsomal pellet was resuspended in Tris–KCl buffer in approximately half the original volume and repelleted at $105{,}000 \times g$ for 1 h. The final microsomal pellet was resuspended in a buffer containing Tris–HCl (0.1 M pH 7.4), glycerol (20%), and stored at −80 °C.

2.4. Clinical pathology

Blood for clinical pathology measurements was collected at necropsies 24 h after administration. Serum enzymes including aspartate amino transferase (AST) and alanine amino transferase (ALT) are used in the evaluation of hepatic disorders.

2.5. Hisopathology

The livers were fixed in 4% paraformaldehyde in 0.1% PB and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin, and were examined under a light microscope.

2.6. Myoglobin digestion

100 μg myoglobin was dissolved in 100 μl 50 mmol/L NH_4HCO_3 (pH 8.0) and denatured in 95 °C water for 5 min before cooling to the room temperature. Trypsin was added to a final protease: protein ration of 1:50 (w/w). The digestion reaction was kept at 37 °C for 12 h. Being divided into two parts equally, the generated peptides were lyophilized and stored at −80 °C before use.

2.7. SDS–PAGE

The appropriate amount of extracted microsomal proteins from normal and CCl_4-stimulating rats were suspended in loading buffer (50 mM/L Tris–HCl pH 6.8, 100 mM/L DDT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), respectively. The samples were run on SDS–PAGE ($T = 13\%$) in Tris–glycine running buffer with 30 μg protein per lane, and stained with Coomassie blue R250.

2.8. In-gel digestion

As the molecular weight of CYP450 proteins were mainly distributed from 45 to 66 kDa, the corresponding gel areas of four lanes were respectively sliced manually as shown in Fig. 2. Further, the excised gel strips were sliced into about 1 mm^3 particles before they were destained with 50% ACN/25 mM NH_4HCO_3, reduced with 10 mM DTT at 56 °C and alkylated in the dark with 50 mM iodoacetamide at room temperature for 1 h. Then the gel plugs were lyophilized and immersed in 15 mL of 10 ng/mL trypsin solution in 25 mM NH_4HCO_3. The digestion was kept at 37 °C for 15 h. Tryptic peptide mixtures were first extracted with 100 mL 5% TFA and then with same volume of 2.5% TFA/50% ACN. The extracted solutions were blended, lyophilized and used for further stable isotopic labeling.

2.9. Guanidination and acetylation

The ε-amino group of lysine was guanidinated according to the previous methods (Everley et al., 2006; Beardsley et al., 2000) with some modifications. 100 μl of 1 mol/L *o*-methyl isourea (dissolved in 0.5 mol/L carbonate buffer, adjusted to pH 11 with 1 mol/L NaOH) was respectively added to the resultant peptides from myoglobin and gel digestion and incubated at 37 °C for 2 h. Then the solutions were adjusted back to pH 8.0 with 1 mol/L HCl for acetylation. Another 200 μl borate buffer was added to the solution for the maintenance of pH value. d0-acetic anhydride and d6-acetic anhydride were diluted into 1 mol/L with tetrahydrofuran. The usage amount of diluted acetic anhydride was 1 μl per 50 μg digested peptides. The acetylation reaction was preceded at room temperature for 1 h. Then *N*-hydroxylamine was used to hydrolyze the esters formed during the acetylation reaction incubating for 30 min (pH 11.0). The serum resultant solutions were desalted by the solid phase extraction column, lyophilized, and stored at −80 °C before use.

2.10. Standard curve

In order to test the linear dynamic range of the stable isotopic labeling method, the myoglobin digested peptides labeled by d0-acetic anhydride and d6-acetic anhydride were diluted into 50, 100, 250, and 500 fmol/μl respectively and mixed in equal aliquot to form a series of ratios (1:10, 1:5, 1:2, 1:1, 2:1, 5:1 and 10:1). Then each 2 μl blended resultant mixtures were loaded and separated by reversed-phase fractionation on a Paradigm MS4B™ HPLC instrument (Michrom BioResources, Inc., Auburn, CA). A 0.2 × 50 mm, 3 μm, 300 Å Magic C8 column, operated at a flow rate of 5000 μl/min. Elution A was 2% acetonitrile, 0.1% FA in water, and elution B was 80% acetonitrile, 0.1% FA in water. The gradient was 40%

B at 10 min, to 100% B at 12 min, to 100% B at 15 min, to 100% A at 16 min, and to 100% A at 18 min. A 7-Tesla LTQ-FTICR mass spectrometer (Thermo Electron, San Jose, CA) was used to record the data. One FT full MS scan was followed by five data-dependent LTQ MS/MS scans on the five most intensive ions. The dynamical excluding time was 15 s. Ions were accumulated in linear ion trap controlled by AGC. The AGC values were 5×10^5 charges for FT full MS scan and 1×10^4 charges for LTQ MS/MS scan. The resolution was 10,000 for FT full MS scan at *m/z* 400. The temperature of the ion transfer tube was set at 200 °C and the spray voltage was set at 1.8 kV. The isolation width was 4 Da and normalized collision energy was 35% for MS/MS scan. The *m/z* range of primary scan is from 400 to 2000.

2.11. Analysis of labeled serum samples using LTQ-FT MS

The labeled CYP450 resultant peptides was analyzed by a 7-Tesla LTQ-FTICR mass spectrometer (Thermo Electron, San Jose, CA) coupled with a Agilent 1100 nano-flow liquid chromatrography system. The reverse phase C18 trap columns (300 μm i.d. × 5 mm long column) were connected with the 6-port column-switching valve for the on-line desalting. A PicoFrit™ tip column (BioBasic C18, 5 μm particle size, 75 μm i.d. × 10 cm long column, 15 μm i.d. at spray tip, New Objective, Woburn, MA, USA) was used for the following separation. Elution solutions were that solvent A was 2% acetonitrile, 0.1% FA in water, and solvent B was 80% acetonitrile, 0.1% FA in water. A gradient was 40% B at 80 min to 100% B at 95 min, to 100% B at 115 min, to 100% A at 120 min, and to 100% A at 130 min. One FT full MS scan was followed by three data-dependent LTQ MS/MS scans on the three most intense ions. The dynamical excluding time was 15 s. Ions were accumulated in linear ion trap controlled by AGC. The AGC values were 5×10^5 charges for FT full MS scan and 1×10^4 charges for LTQ MS/MS scan. The resolution was 10,000 for FT full MS scan at *m/z* 400. The temperature of the ion transfer tube was set at 200 °C and the spray voltage was set at 1.8 kV. The isolation width was 4 Da and normalized collision energy was 35% for MS/MS scan. The *m/z* range of primary scan is from 400 to 2000.

2.12. Data processing and analysis

The acquired raw file was converted to *.dta files by BioWorks 3.2 and all the *.dta files were merged into one *.mgf format file by a script named merge.pl. Then the *.mgf file was searched against IPI rat 3.15 (ftp://ftp.ebi.ac.uk/pub/databases/IPI/old/RAT/ipi.RAT.v3.15.fasta.gz) through local MASCOT (Version1.9) with 95% confidence. Because the same peptides with differential N-terminal modifications may be fragmented, the dataset was searched twice using two sets of parameters. In the first set of parameters, the N-terminal fixed modification was d0-acetylation (+42.0106 Da, the peptide was labeled with the light chain reagent), and the second was d3-acetylation (+45.0294 Da, the peptide was labeled with the heavy chain reagent). Other parameters were same: fixed modifications including cysteine carbamidomethyl (+57.0214 Da), lysine guanidination (+42.0218 Da) were set, full tryptic cleavage was selected, and the maximal allowable miss-cleavage sits was 2. Mass tolerance for peptide and fragment ions was 5 ppm and 1.2 Da respectively. Based on the mascot results, the monoisotopic peaks' ratio between the heavy labeled peptides and light labeled peptides was automatically calculated by a graphic user interface program called 'MSAQ' developed in house.

2.13. Statistical analysis

Means and standard errors of individual groups were calculated. And the difference between means was compared using least-squares difference (LSD) at 0.05 significant level.

3. Results

3.1. Blood biochemistry and histopathology

An increase in these enzyme activities reflects active liver damage. CCl_4 treatment (1 ml/kg, ip) resulted in marked increases of serum ALT and AST activities (Table 1), which were significantly higher than those of the control groups.

Histopathological findings after the treatment of CCl_4 are summarized in Fig. 1. Histopathology of Group B animals (CCl_4-induced group) showed patches of liver cell necrosis with inflammatory collections around central vein.

3.2. SDS–PAGE

A 1-D electrophoresis pattern of total proteins from two types of rat liver microsomes is presented on Fig. 2. The normal sample was remarked with A, and the CCl_4-stimulating sample was remarked with B. As we can see, there was no apparent change in banding on the gels. This may be due to the low resolution of the gels.

Table 1
Carbon tetrachloride related change on the activity of amino transferases (ALT and AST), in the liver of male rats

Control	CCl_4
ALT 32.4 ± 0.47	137.5 ± 7.3*
AST 26.8 ± 3.0	186.0 ± 12.0*

Note: (*) refers to statistical difference ($P < 0.05$). Values are the mean ± S.E. of four male rats of each group. ALT, AST activity expressed as international unit (IU), which is defined as the amount of the enzyme that under defined assay conditions would catalyze 1 mol of substrate/min/l.

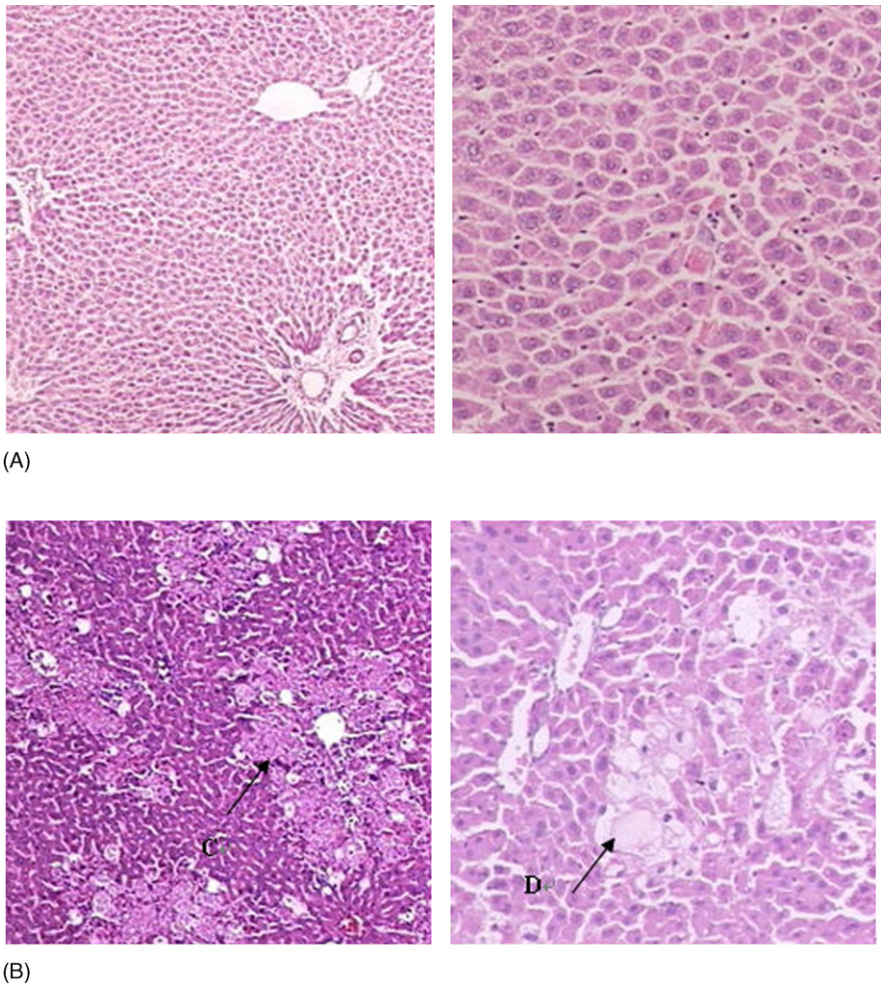

Fig. 1. Histopathologic change of male rat liver: (A) liver of control guoup. Normal centrilobular region were shown. (B) Liver of CCL_4 induced group. Some centrilobular hepatocyte necrosis and microvesicular fatty change were shown; part (C) shows typical central are a of hepatocllular necrosis; part (D) shows microvesicular fatty change.

3.3. Stable isotopic labeling method (GA labeling)

It was a simple labeling method and mainly composed of two steps. First ε-amino group of lysine was guanidinated (G), and then the N-terminal amino group was acetylated by d0/d3-acetic anhydride (A). Guanidination reaction could convert lysine into homoarginine with a mass shift of 42.0218 Da. So only the N-terminal amino group would be acetylated. At the same time, only one acetyl group would be incorporated into per peptide. If ε-amino group of lysine was not blocked, it was also easily acetylated by acetic anhydride in the same reactive condition. Once the above events emerged, the mass difference of peptides between the light reagent labeled and the heavy reagent labeled would not be a constant (mass difference is 3.0188 Da in +1 charge state). More deuterium atoms incorporated into peptides would dramatically result in serious isotopic effect during the reverse phase chromatography separation (Zhang et al., 2002). Thus, the ionizing efficiency of various labeled peptides would not be the same and the quantitative results would not be correct and reliable. Fig. 3 showed the ratio calculation method between the heavy labeled peptide and the light labeled peptide.

3.4. Standard curve

From Fig. 4, we see a perfect correlation between theoretical and observed ratios and the correlation coefficient was 0.9998 during the 100-fold dynamic range (from 10:1 to 1:10). At the same time, the ratio error was only 4.2%. The above results showed that GA labeling method is highly reliable for quantitative analysis at 100-fold dynamic range.

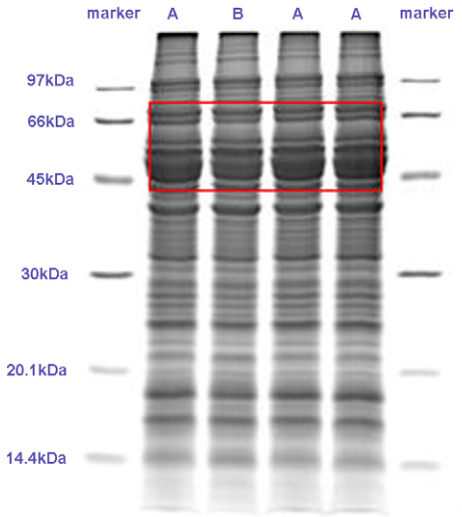

Fig. 2. 1-D electrophoresis pattern of total proteins from two types of rat liver microsomes. The normal sample was remarked with A, the CCl_4-stimulating sample was remarked with B. The molecular weight of CYP450 proteins are mainly distributed from 45 to 66 kDa as shown in the red square above.

3.5. Scheme of relative quantification analysis of cytochrome P450 expression changes

As Fig. 5 showed, after SDS–PAGE, digestion and extraction, two normal samples (A) among four lines were labeled by light reagent and heavy reagent respectively, and then they were analyzed by RP-LTQ-FT MS with two runs repeat. As the same sample were labeled, it was easy to determine the distribution of D/H ratios in the control sample A + A (Fig. 6), so was in the experiment sample of A + B.

In all, 17 CYP proteins were identified and quantified during the control sample A + A with two runs

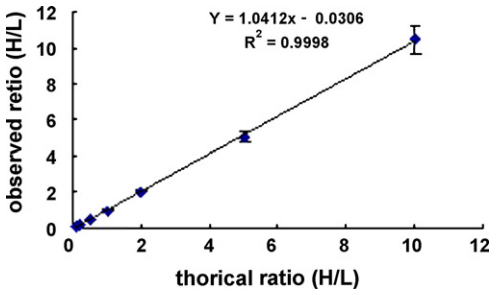

Fig. 4. The dynamic linear range of the labeling method. Each spot stands for the average value of six labeled peptides, and the error bar is standard deviation.

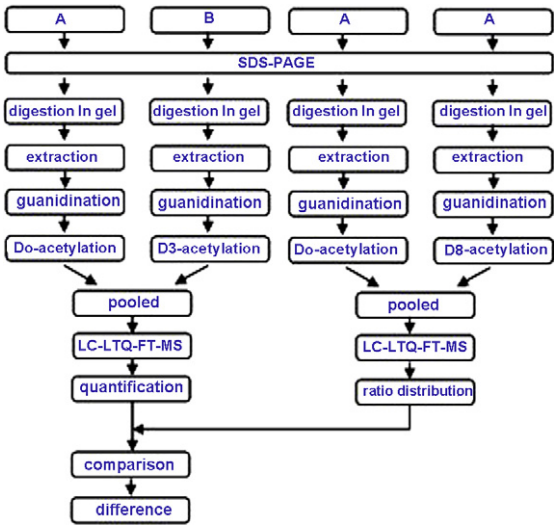

Fig. 5. Scheme of CYP450 expression profiling quantitative analysis. Sample A is from normal rats, and sample B is from CCl_4-stimulating rats. Four lines (A, B, A, and A) are with the same loading amounts (30 μg).

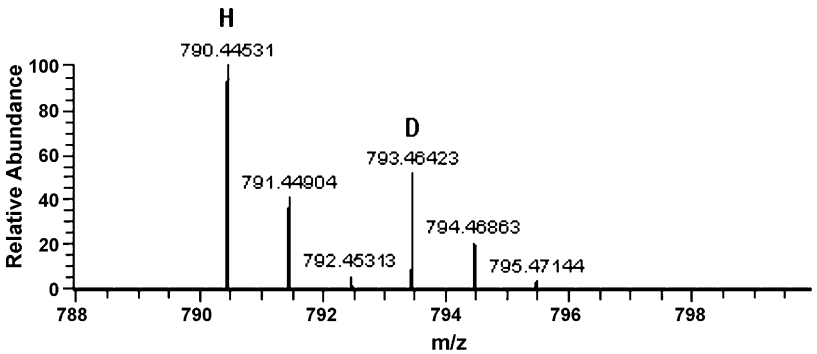

Fig. 3. FTICR-MS quantitative analysis of a peptide (ALELFR) mixture labeled by GA method. The peak pair of labeled peptide combined with a D/H molar ratio of 1:2. The peak at 790.4453 Da corresponds to the D3-acetylation form of the singly protonated monoisotopic peptide. The peak at 793.4642 Da corresponds to the H3-acetylation form of the singly protonated monoisotopic peptide. The D/H ratio of the combined monoisotopic peak intensities was 0.51.

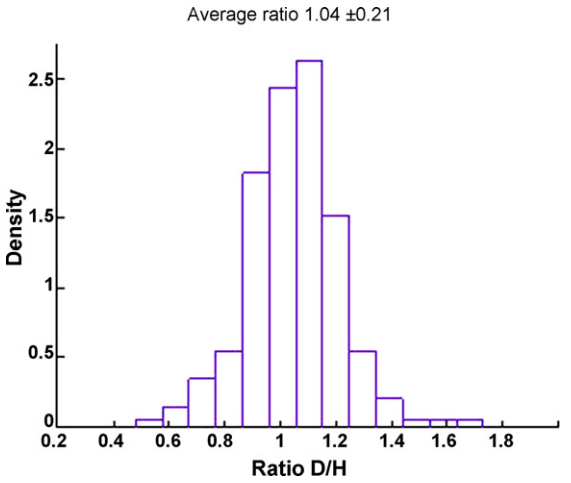

Fig. 6. Distributions of D/H ratios in the control sample A + A. The average ratio was 1.04. The standard deviation was 0.21.

repeats. The average ratio was 1.04 and very close to theoretical value 1.00. The standard deviation was 0.21. If the abundance ratios differ from 1.0 with more than three-fold S.D. (0.21) from the control experiment (either >1.67 or <0.59), the proteins are considered to have significant changes in abundance following CCl_4 stimulating.

Because CYP450 was a super family, the family classification was performed further. From the Fig. 7, the distribution of our identified CYP450 proteins was similar to the distribution in the dataset (IPI3.15rat). In it, family 2 is a main family and includes more CYP450 proteins. In our results, the number of CYP450 proteins in family 2 was still the largest among all the families.

In order to make the quantitative results more reliable, all proteins identified in two runs were selected for quantitative analysis. 17 CYP450 proteins were quantified confidently. Table 2 shows the details.

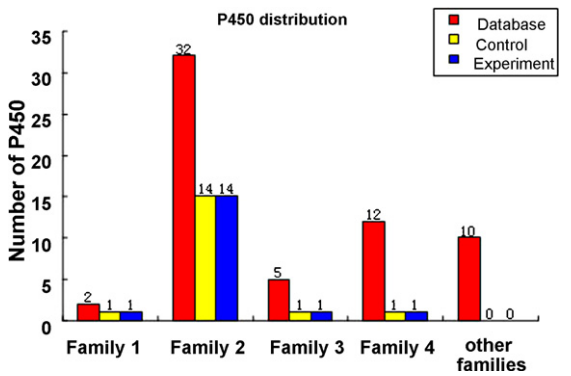

Fig. 7. Distribution of the identified CYP450 proteins both in our experiment and in the database (IPI3.15rat).

4. Discussion

The aim of the present study was to investigate the profile of CYP isoforms protein of CCl_4-induced liver in male rats by proteomic approach. It is well known that carbon tetrachloride exposure can damage the liver and kidneys, and is a rodent hepatocarcinogen. The results of the present study demonstrate that CCl_4 treatment of rats markedly improved marker serum (namely serum ALT and AST) and caused liver damage, as confirmed by biochemical assays and histopathological examination.

From previous studies, carbon tetrachloride was found to be metabolized by CYP-dependent manners in rat liver microsomes. In our study, it is demonstrated that in the male rat, CCl_4 induced protein expression of some but not all CYP450 isoforms were regulated at different levels. In total, 17 CYP proteins were identified in male rat livers. Among them, the expression amount of 2C11, 3A2, and 2 E1 were down-regulated, while that of 2C6, 2B2, and 2B1 were up-regulated in the CCl_4 induced group.

CYP2E1 is a major constitutive enzyme of mammalian liver with critical roles in xenobiotic metabolism, toxicity, and carcinogenesis (Ronis et al., 1996). Although CCl_4-induced liver damage that occur at morphological, histopathological and biochemical levels remain unclear, it is clearly demonstrated that CYP2E1 plays an important role in CCl_4-induced liver damage. It is known that CCl_4-induced hepatotoxicity is caused by free radicals which are metabolic products of CCl_4 mainly formed by CYP2E1. CYP2E1 has a high capacity to generate free radicals (Ingelman-Sundberg and Johansson, 1984) which, in turn, stimulate lipid peroxidation. In this study, we observed a decrease in CYP2E1. In agreement with the present study, it has been reported that CYP2E1 protein was reduced after CCl_4 exposure (Wong et al., 1998; Sinclair et al., 1991). Our present observation can be well explained by the mechanism of the degradation of CYP2E1 (suicide metabolism). CCl_4 metabolism is a suicidal reaction — a reactive metabolite formed from this chemical degenerating or inactivating the isozyme. The target isozyme for this in-activation is CYP2E1. Almost all of the labilized CYP2E1 protein is immediately degraded and removed from the endoplasmic reticulum.

Several other mechanisms have also been suggested for CYP2E1 degradation. Previous studies have indicated that the rapid degradative pathway appears to be regulated by hormone-regulated phosphorylation of CYP2E1 (Eliasson et al., 1990, 1992; Johansson et al., 1991). It was also suggested that an ubiquitin-dependent degradation pathway could be involved in the rapid

Table 2
Relative abundance changes

Accession ID	Description	Protein				Peptide sequence	Peptide	
		Ratio 1	Ratio 2	Average	S.D.		Ratio 1	Ratio 2
						DIGQSIK	0.19	0.2
						NYFIPK	0.25	0.24
						NFFYIK	0.26	0.21
						VQEEIER	0.21	0.23
IPI00327781.1	2C11	0.36	0.32	0.34	0.03	DFIDCFLNK	0.93	0.84
						FDPGHFLDER	0.23	0.21
						EHQESLDKDNPR	0.24	0.22
						YIDLVPTNLPHLVTR	0.19	0.21
						IQEEAQCLVEELRK	0.7	0.68
IPI00196764.1	3A2	0.36	0.33	0.35	0.02	QAILEPEKPIVLK	0.36	0.33
						LHEEIDR	0.64	0.78
IPI00324912.5	2E1	0.58	0.52	0.55	0.04	VVLHGYK	0.56	0.47
						FINLVPSNLPHEATR	0.55	0.51
						FSLSILR	–	0.32
						NYLIPK	1.62	1.57
						NVYHIR	2.33	–
						DLGIVFSHGNR	1.25	1.48
						EHQESLDVTNPR	1.94	2.24
IPI00390958.2	2C6	1.72	1.75	1.74	0.02	EALIDHGEEFAER	2.56	2.83
						FTLTTLR	1.99	1.9
						DFIDYYLIK	1.94	2.09
						GTTIITSLSSVLHDSK	0.7	0.82
						FIDLIPTNLPHAVTCDIK	1.19	1.2
						IKEHQESLDVTNPR	–	1.61
IPI00193234.1	2B2	4.21	3.55	3.88	0.47	LLELFYR	4.21	3.55
IPI00206969.1	2B1	4.11	4.02	4.07	0.06	DIDLTPK	4.01	4.48
						LLELFYR	4.21	3.55
						FSVSTLR	1.24	1.23
						FQGFLIPK	0.86	0.84
						AVSNVIASLVYAR	0.69	–
IPI00231473.4	2D26	1.04	1.07	1.06	0.02	VHEEIDEVIGQVR	1.12	1.2
						MPFTNAVIHEVQR	1.34	1.12
						EAEHPFNPSILLSK	0.98	1.01
						FHPEHFLDAQGNFVK	–	1.03
						YPHVTAK	1.08	0.95
						VQEEIDHVIGR	0.66	0.72
						EALVDHGEEFSGR	0.67	0.64
IPI00325874.1	2C13	0.93	0.78	0.86	0.11	IKEHEESLDVSNPR	1.42	1.14
						FDYEDKDFLNLIEK	0.81	0.7
						GTAVLTSLTSVLHDSK	0.92	0.91
						IKEHEESLDVSNPR	–	0.41
						FGDIAPLNLPR	1.34	1.46
IPI00202584.1	2D10	1.48	1.45	1.47	0.02	NLTDAFLAEVEK	1.29	1.11
						VQQEIDEVIGQVR	1.73	1.84
						RFEYEDPYLIR	1.58	1.39
						DFVDYYLIK	1.43	1.11
						VLTSLTSVLHDSK	1.02	1.02
IPI00196748.3	2C7	1.11	1.18	1.15	0.05	YPHVTAK	1.08	0.95
						IEEHQESLDVTNPR	0.93	1.11
						VQEEAQCLVEELRK	–	1.7
						LSIATLR	1.07	1.06
						VHEEIEQVIGR	1.06	1.17
IPI00196696.5	2A1	0.99	1.09	1.04	0.07	QNHSTLDPNSPR	0.68	0.98
						ILEEAGYLIK	1.29	1.48
						TVSNVISSIVFGER	0.84	0.66
						LEDINESPKPLGFTR	–	1.16

Table 2 (*Continued*)

Accession ID	Description	Protein				Peptide sequence	Peptide	
		Ratio 1	Ratio 2	Average	S.D.		Ratio 1	Ratio 2
IPI00327991.3	2C23	1.38	0.92	1.15	0.33	LPYTDAVLHEIQR	1.38	0.99
						NFILEK	–	0.96
						YITLLPSSLPHAVVQDTK	–	0.5
						FDPGHFLDK	–	1.25
IPI00198324.1	2B3	0.97	1.01	0.99	0.03	LLDLLYR	0.89	0.94
						EALVDHAEAFSGR	1.18	1.14
						MPYTEAVIHEIQR	0.83	0.96
						IHEELDTVIGR	0.61	–
IPI00198947.1	1A2	0.82	0.69	0.76	0.09	TVQEHYQDFNK	0.88	0.69
						NPHLSLTK	0.98	–
IPI00203317.1	4A2	1.40	0.99	1.20	0.29	KAQLQNEEELQK	1.4	0.99
IPI00324707.7	2C12	1.62	1.61	1.62	0.01	DIDINSIR	1.62	1.61
IPI00393883.2	2C70	0.70	0.62	0.66	0.06	KTTQDVEFR	0.70	0.62

If the abundance ratios differ from 1.0 with more than three-fold S.D. (0.21) from the control experiment (either >1.67 or <0.59), the proteins are considered to have significant changes in abundance following CCl_4 stimulation. Proteins with only one unique peptide detected were considered to be less confidently quantified than those proteins with multiple peptides detected.

degradation of CYP2E1 labilized by CCl_4 (Tierney et al., 1992).

CYP2E1-mediated metabolism of CCl_4 generated reactive free radicals, and CYP2E1 protein might be more susceptible to CCl_4 toxicity than other CYP isozymes. Many previous investigations regarding CCl_4-induced liver injury have focused only on CYP2E1 activity, not on other CYP isoforms (Yokogawa et al., 2004; Jeong et al., 2002; Jeon et al., 2003). As shown in the results from our study, the level of CYP2C11 and CYP3A2 were also decreased by CCl_4-treatment. Rat liver contains at least a dozen of sex-dependent isoforms of CYP. Both CYP2C11 and CYP3A2 are sexually dimorphic liver enzymes, the expression of which is regulated by the male pulsatile release pattern of growth hormone (GH) (Agrawal and Shapiro, 2000; Timsit and Riddick, 2000).

CYP2C11 is a major isoenzyme in the liver of male rats. This isoenzyme accounts for about one third of the total cytochrome P450 in S.D. male rat liver and it metabolizes a host of xenobiotics such as benzphetamine, aminopyrine, ethylmorphine, benzopyrene and warfarin (Chen and Ronis, 1997). GH secretion might be very sensitive to environmental stress. Moreover, it was found that the response of the liver of the GH-suppressed Mini rats to CCl_4 was different from that of the Wistar rats. (Shimizu et al., 2001). Our results suggest that CCl_4 may interfere with the ability of GH to regulate CYP2C11 expression in male rats. Expression of CYP3A2, another male-specific isoform, was observed to have down-regulated in the present study. Like CYP2C11, CYP3A2 expression can be suppressed by changes in the masculine growth hormone profile characterized by shortened interpulse durations.

Between rat CYP2B1 and CYP2B2 proteins there is only a 13 amino acid difference, mostly in the C-flanking sequence of the protein (about 3% of the total CYP sequence), though CYP2B1 and CYP2B2 genes are nonallelic variants of the gene which contains nine exons (Gonzalez, 1989). In our study, we not only successfully identified CYP2B1 and CYP2B2, but also observed an up-regulation in both CYP2B1 and CYP2B2 by similar amount.

In spite of the important physiological and pharmacological roles of CYP2C6, the enzyme has not been fully investigated so far in CCl_4-induced male rats. In the liver of male rats, the contribution of CYP2C6 isoform to the total CYP content is approximately 20%. Rat CYP2C6 can be regarded as a counterpart of human CYP2C9 (Gerbal-Chaolin et al., 2001). Our study showed that CYP2C6 was up-regulated upon treatment with CCl_4. CYP2B1/2 and CYP2C6 might be involved in the detoxification. This study demonstrates that change in CYP2E1 may not be of only consequence in CCl_4-induced liver damage and that other P450s should be considered.

CYP1A1, CYP1A2, CYP2B (specifically CYP2B1 and CYP2B2), CYP2E1, and CYP3A (specifically CYP3A1 and CYP3A2) are important in xenobiotic metabolism, activation, and detoxification as well as in the metabolism of endogenous compounds. Although there is a previous report on down-regulated CYP1A1 expression in male rat hepatocytes as a result of oxidative stress (Laville et al., 2004), our results failed to detect

CYP1A1 protein in liver microsomes. This may be that CYP1A1 is expressed at very low levels in hepatic. Our study results showed no change in CYP1A2.

In this paper, we provide a rapid, high throughput approach to relative quantitative analysis which may be helpful in toxicology research. This approach to monitoring changes in P450 isoenzymes has many advantages. Firstly, it has the potential to discriminate between closely related isoforms. Secondly, it may be an effective way to reveal as-yet-unknown enzyme changes while lacking the specificity for determining enzyme subtype expression. Thirdly, the overall P450 protein expression profiles in the *in vivo* hepatoxicity of male rats induced by CCl_4 can be well summarized.

In conclusion, the findings of this study clearly demonstrate that it is fairly possible to use comprehensive protein expression profiles approach in toxicological evaluation.

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