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Subproteomic Analysis of the Mitochondrial Proteins in Rats 24 h After Partial Hepatectomy

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ABSTRACT

A 70% partial hepatectomy (70%PHx) induces cell proliferation until the original mass of the liver is restored. Mitochondria are involved directly in the process of liver regeneration (LR); however, their role in the early phase of LR is not clear. In an attempt to identify mitochondrial proteins that are correlated with the early phase of LR, we obtained a mitochondrial fraction via Nycodenz® density gradient centrifugation and subcellular proteomic analysis was performed. The mitochondrial proteins were separated by two-dimensional gel electrophoresis and identified by mass spectrometry. Compared to the sham-operation control group, 3 proteins were up-regulated and 22 proteins were down-regulated at 24 h after 70%PHx. We identified 22 differentially expressed proteins that were associated with carbohydrate metabolism, lipid metabolism, the respiratory chain and oxidation–phosphorylation, biotransformation and other metabolic pathways. Prohibitin, a proliferation-regulating protein that was down-regulated at 24 h after PHx, was analyzed by applying RNAi (PHBi) with BRL-3A. This demonstrated a decreased mitochondrial membrane potential, implying a potential role in maintaining mitochondrial integrity. These results indicated that hepatic mitochondrial adaptations to LR after 70%PHx affect various cellular metabolic pathways, which advances our knowledge of the role of mitochondria in the early phase of LR. J. Cell. Biochem. 105: 176–184, 2008.

KEY WORDS: SUBCELLULAR PROTEOME; PARTIAL HEPATECTOMY; MITOCHONDRIA; TWO-DIMENSIONAL GEL ELECTROPHORESIS; MASS SPECTROMETRY; PROHIBITIN

A 70% partial hepatectomy (70%PHx) induces cell proliferation until the original mass of the liver is restored [Michalopoulos, 1990]. Acceleration of liver regeneration (LR) could be of great clinical benefit in various liver-associated diseases. However, because of our limited understanding and the complexity of the mechanisms involved in LR, at present little is known about therapeutic interventions to enhance this regenerative process.

The expression of clusters of differentially regulated genes in the process of LR has been described. However, studies should not be limited to post-translational gene regulation but should include the effect of post-transcriptional regulatory mechanisms, which alter the abundance and function of the corresponding gene products [Gygi et al., 1999; Chen et al., 2002]. Details of the mechanism underlying the control of LR are not known. With the development and establishment of 2-DE and MS technology, the protein expression pattern of rat liver and LR was focused on [Fountoulakis and Suter, 2002]. Subcellular fractionation and purification of organelles are valuable additions to the protein separation techniques commonly used in proteomic analysis.

Mitochondria are complex subcellular organelles with key roles in many cellular functions, including energy production, calcium homeostasis, cell signaling and aging [Thress et al., 1999; Newmeyer and Ferguson-Miller, 2003; Melov, 2004]. In the light of early changes in ATP concentration found in liver after PHx, before activation of cell proliferation [Ngala-Kenda et al., 1984], mitochondria were investigated as they are involved directly in LR [Inomoto et al., 1994; Guerrierie et al., 1995, 1999; Vendemmiale et al., 1995]. However, details of the underlying mechanism are not known.

In this study, subcellular proteomics were used in combination with subcellular fractionation to analyze alterations in the mitochondrial proteome in rats at 24 h after 70%PHx. The differences in expression of proteins indicated that hepatic mitochondrial adaptation to LR after PHx affects various cellular metabolic pathways, such as carbohydrate metabolism, respiratory chain and oxidation–phosphorylation, and lipid metabolism. The proliferation-regulating protein prohibitin was down-regulated, and analysis by RNAi (PHBi) with BRL-3A demonstrated decreased mitochondrial

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membrane potential, implying a role in the maintenance of mitochondrial integrity.

MATERIALS AND METHODS

ANIMALS AND SURGERY

Adult male Sprague–Dawley rats (220–250 g) were obtained from the Experimental Animal House at the Second Military Medical University (Shanghai, China). They were divided at random into two groups: five were used as the sham-operation control group and another five were used as the 70%PHx test group. All experiments were done in triplicate. Both groups were housed with a 12 h light/12 h dark cycle, and given a standard diet until they were euthanized. PHx (−70%) was done in the test group as described [Higgins and Anderson, 1931]; the median and left lateral lobes of the liver were removed without injuring the remaining tissue. The control group was subjected to a sham operation with the same procedure as the test group but without liver removal.

NYCODENZ® DENSITY GRADIENT CENTRIFUGATION AND MITOCHONDRIAL PROTEIN PREPARATION

Subcellular fractionation of rat liver and Nycodenz® density gradient centrifugation were done essentially as described but with minor modifications [Ayako and Fridovich, 2002]. Nycodenz® was dissolved to 50% (w/v) in 5 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA and a mixture of protease inhibitor (1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.2 mM Na_3VO_4, 1 mM NaF). This stock solution was diluted with 5 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 0.5 mM EGTA, and a mixture of protease inhibitor and phosphatase inhibitors. The crude mitochondrial pellets obtained from differential centrifugation [Jiang et al., 2004] were suspended in 12 ml of 25% Nycodenz® and placed onto a discontinuous Nycodenz® gradient consisting of 5 ml of 34% Nycodenz® and 8 ml of 30% Nycodenz®, followed by 8 ml of 23% Nycodenz® and finally 3 ml of 20% Nycodenz®. The sealed tubes were centrifuged for 90 min at 52,000*g* at 4°C. The mitochondria were in the band at the 25%/30% interface, which was collected and diluted with the same volume of homogenization buffer and then centrifuged twice at 15,000*g* for 20 min. The samples of mitochondrial protein were prepared as described [Jiang et al., 2004]. The mitochondria pellets from Nycodenz® density gradient purification were suspended in lysis buffer (8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), 65 mM dithiothreitol (DTT), 40 mM Tris), sonicated at 100 W for 30 s, and centrifuged at 25,000*g* for 1 h. The supernatant was collected and the protein concentration was determined with a Quick Start Bradford Assay Kit (Bio-Rad).

2-DE, GEL STAINING, AND TRYPTIC DIGESTION OF 2-DE GEL SPOTS

The 2-DE was done essentially according to the manufacturer's protocol. Briefly, samples of mitochondrial protein (0.2 mg) were added to 0.35 ml of rehydration solution (8 M urea, 4% (w/v) Chaps, 65 mM DTT, 0.2% (w/v) carrier ampholytes and a trace of bromophenol blue). For the first dimension, the sample was applied to immobilized 17 cm, pH 3–10 nonlinear IPG strips (Bio-Rad). Rehydration was done at 50 V for 14 h. Isoelectric focusing (IEF) using a Proten IEF Cell apparatus (Bio-Rad) was started at 250 V and the voltage was increased gradually to 10,000 V and kept constant until 70,000 Vh. The second dimension electrophoresis was carried out on 10% SDS–PAGE gels (230 mm × 200 mm × 1.5 mm) at a constant current of 25 mA/gel. The 2D gels were silver-stained as described [Shevchenko et al., 1996]. To reduce error, we improved the second dimension electrophoresis by cutting the strips gently from the middle point after IEF, and placing the acidic extremity and the alkaline extremity of the control sample and the test sample, respectively, on the same SDS–PAGE gels. After electrophoresis the gels were scanned with an ImageScanner (UMAX). Spot detection, spot matching, and quantitative intensity analysis were performed using PDQuest7.4 software (Bio-Rad). The gel images were normalized according to the total quantity in the analysis set. The difference in the abundance of differential protein spots was analyzed with Student's *t*-test. Any difference with a *P*-value ≤ 0.05 was considered statistically significant. The silver-stained protein spots were digested as described [Gharahdaghi et al., 1999].

MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT (MALDI-TOF) MASS SPECTROMETRY (MS) AND DATABASE SEARCH

Each sample was suspended in 0.7 μl of matrix solution (α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile/water (1:1, v/v) acidified with 0.1% (v/v) TFA). The mixture was immediately spotted onto the MALDI target and allowed to dry and to crystallize. The analyses were performed on a 4700 Proteomics Analyzer (TOF/TOF™) (Applied Biosystems, USA) equipped with a 355 nm Nd:YAG laser. The proteins were identified by peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS) using the program MASCOT v. 1.9 (Matrix Science, London, UK) against SWISS-PROT database with GPS explorer software (Applied Biosystems). MASCOT protein scores (based on combined MS and MS/MS spectra) of greater than 61 were considered statistically significant ($P \leq 0.05$).

CELL CULTURE

The normal rat liver cell line BRL-3A was obtained from the Shanghai Institute of Biochemistry and Cell Biology. The BRL-3A cells were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were maintained at 37°C in an atmosphere with 5% CO_2.

RNA INTERFERENCE

Duplex siRNA was obtained from GeneChem (Shanghai, China). The siRNA sequence targeting rat prohibitin was 5′-GCCAGAUUUGUG-GUGGAAAtt-3′ (sense) and 5′-UUUCCACCACAAAUCUGGCtt-3′ (antisense). A nonsense duplex was used as the control. BRL-3A cells were plated on 6-well plates with antibiotic-free DMEM overnight and transfected with siRNA by Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. The final concentration of siRNA duplex was 100 nM. Six hours after transfection, the medium was switched to DMEM supplemented with antibiotics.

RT-PCR AND QUANTITATIVE REAL-TIME PCR

Cells or homogenized liver tissue were harvested with Trizol reagent (Invitrogen) according to the manufacturer's protocol. After treatment with DNaseI, each RNA sample was reverse-transcribed with random primers (dN_6) following the manufacturer's protocol (MBI Fermantas, Vllnlus, Lithuania). The single-stranded cDNA was used in quantitative real-time PCR to evaluate the relative expression levels of PHB (5′-GCGGTGGAAGCCAAACAG-3′ and 5′-TTCTTCTGCTGCTCAGCCTTT-3′), compared to β-actin (5′-ATGGTGGGTATGGGTCAGAAG-3′ and 5′-TGGCTGGGGTGTTGA-AGGTC-3′) used as an internal control for determining cell number and metabolic status. Quantitative real-time PCR (ABI7300, Applied Biosystems) was done with SYBR Green I reagents (TOYOBO) and the primers were designed according to the ABI manufacturer's protocol. A total of 40 cycles of PCR were performed with 15 s at 95°C, 60 s at 60°C. The real-time PCR signals were analyzed with LightCycler 3.5 software (Roche Diagnostics).

WESTERN BLOTTING

Cells were harvested with trypsinization and rinsed twice in ice-cold PBS, and lysed in sodium dodecyl sulfate (SDS) lysis buffer with protease inhibitor PMSF for 20 min on ice. The samples were boiled and clarified by centrifugation at 12,000*g* for 10 min at 4°C. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane. The blots were probed by anti-prohibitin antibody (Neomarker) and proteins were normalized with anti-β-actin antibody (Neomarker) and visualized with DAB or the Amersham ECL system. The digital image was obtained by scanning the membrane and gray value analysis.

ASSESSMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL

Serum cell samples (3×10^5) were stained with 1 g/ml of JC-1 (5, 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Molecular Probes). After incubation for 10 min in a CO_2 incubator, cells were washed with PBS, and cell suspensions were prepared for flow cytometry essentially as described [Fulda et al., 1998]. Flow cytometry was performed on a FACSCalibur instrument and analyzed with CellQuest software (BD Bioscience).

STATISTICAL ANALYSIS

The data are expressed as mean ± SD of three experiments. Statistical significance was estimated with Student's *t*-test for unpaired observations. A *P*-value of less than 0.05 was considered significant.

RESULTS

COMPARISON OF 2-DE PATTERNS AND DIFFERENTIAL EXPRESSION OF MITOCHONDRIAL PROTEINS

A purified mitochondrial fraction was obtained by Nycodenz® density gradient centrifugation and the proteins derived from hepatic mitochondria were separated on pH 3–10NL IPG strips followed by electrophoresis through 10% SDS–PAGE gels to examine the mitochondrial proteins correlated with the early phase of LR. A total of 350 ± 42 and 272 ± 25 spots were detected in the control and the test group gels, respectively, using PDQuest7.4 software. A total of 200 ± 33 spots in the test group matched with spots in the control group, a matching rate of 73(±8)%. The expression patterns of the mitochondrial proteins showed that most of the protein spots were concentrated in the pH 7–10 region (Fig. 1A). The differences in protein profiles between the control group and test groups were detected by image analysis. Compared with the control group, 25 protein spots in the test group gels (Fig. 1B) displayed a significant and ≥2-fold change in abundance, of which 22 were down-regulated (1–22) and 3 were up-regulated (23–25).

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED MITOCHONDRIAL PROTEINS AND BIOINFORMATICS

The proteins were in-gel digested with trypsin, extracted as peptides and identified by MALDI-TOF/TOF MS and database searching. For each spot, PMF was first done to assign a spot to a protein and then an MS/MS fragmentation was used to confirm the PMF results. We identified 22 differentially expressed proteins (Table I) that were involved in various metabolic pathways, including carbohydrate metabolism, lipid metabolism, and respiratory chain and oxidation–phosphorylation, biotransformation. Prohibitin (Fig. 2; Fig. 1B, spot 16), a proliferation-regulating protein, was down-regulated at 24 h after 70%PHx. Unidentified spots were considered to be protein mixtures and were separated and identified further.

VALIDATION OF DIFFERENTIAL PROTEIN BY QUANTITATIVE REAL-TIME PCR AND WESTERN BLOTTING

All of the differentially expressed proteins in this study were defined by measuring the density values of the protein spots and using an expression change rate threshold equal to or above twofold. To verify whether the differential proteins in our study were real differential ones, we used quantitative real-time PCR and Western blotting for confirmation. We selected prohibitin (Fig. 2; Fig. 1B, spot 16) whose expression change was relatively less obvious compared to other differential proteins to be validated, so that the confirmation results would better assure the liability of our 2-DE data for the other differential proteins with higher expression changes. Figure 3 showed the quantitative real-time PCR and Western blotting results, revealing about a twofold down-regulation of prohibitin mRNA (Fig. 3A) and protein expression (Fig. 3B) in the 70%PHx test group compared to the sham-operation control group. These data agreed with the expression changes shown by the 2-DE analysis.

PROHIBITIN siRNA (PHBi) LEADS TO DECREASED MITOCHONDRIAL MEMBRANE POTENTIAL

Recently, prohibitin was reported to be essential for normal mitochondrial development, and prohibitin deficiency was shown to be associated with deficient mitochondrial biogenesis [Artal-Sanz et al., 2003]. We asked whether the decrease of prohibitin by PHBi had any effect on mitochondrial membrane potential. PHBi showed a dramatic reduction of prohibitin mRNA (Fig. 4A) and protein level (Fig. 4B) compared with that of the control group (mock). Mitochondrial membrane potential was examined by flow cytometry using a mitochondria-specific probe (JC-1). When the mitochondrial membrane potential was high, JC-1 formed J-aggregates that emitted red fluorescence. In contrast, when the mitochondrial

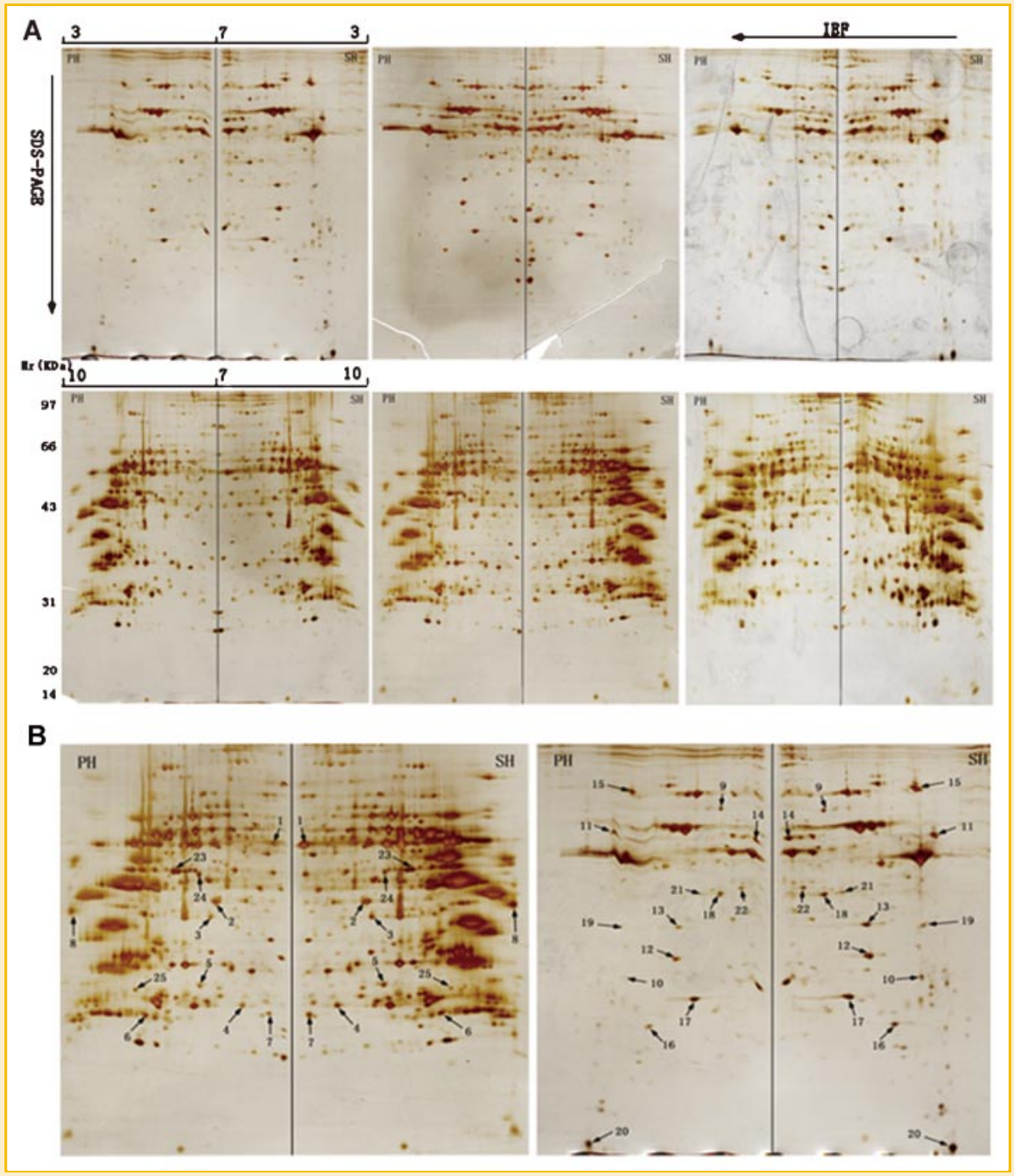

Fig. 1. Patterns comparison of 2-DE and differential expression of mitochondrial proteins. A: Three paired of 2-DE maps of mitochondrial proteins in rat liver. Mitochondrial proteins (0.2 mg) were loaded on pH 3–10 nonlinear IPG strips (17 cm), with 10% SDS–PAGE as the second dimension. The gel was visualized by silver staining. For second dimension electrophoresis, the strips were cut gently from middle point after IEF. Three maps in the upper panel are from the region of pH 3–7; the other three maps in the bottom panel are from the region of pH 7–10. B: Twenty-five differentially expressed proteins were found. The left spectrum is from the region of pH 7–10 and the right is from the region of pH 3–7. Differential proteins are marked by an arrow and number. SH: sham-operation control groups; PH: 70%PHx groups. Three independent repetitions were performed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

membrane potential collapsed, for example, because of membrane damage, these aggregates cannot form and the fluorescence shifts from red to green. Cells with the level of prohibitin decreased by PHBi had a decreased mitochondrial membrane potential (Fig. 4C). In the control group, the mean red fluorescence intensity of 1×10^4 cells was 431.7 ± 62.9, but for PHBi it was reduced to 271.4 ± 37.6.

DISCUSSION

LR involves various molecular events and mitochondria are directly involved. Using subcellular proteomic analysis, the present study showed alterations of rat liver mitochondrial proteome in the early phase of LR induced by 70%PHx. Changes of mitochondrial

TABLE I. Identification of the Differentially Expressed Mitochondrial Proteins

No.	NCB Inr index code	Protein name	Protein score	Mr (KDa)	pI	MS/MS	Note
1	gi|45737866	Mitochondrial aldehyde dehydrogenase precursor (rat)	241	55566.2	6.69	AAFQLGSPWR/TS YTR/TFVQEDVYDEFVER	$P < 0.05$
2	gi|48734846	Acyl-coenzyme A dehydrogenase, short chain (rat)	116	44939	8.47	LVIAGHLLR/LHTVELPETHQML	$P < 0.05$
3	gi|205892	Preornithine carbamoyl transferase (rat)	74	39930.8	9.12	KPEEVDDEVFYSPR	$P < 0.05$
4	gi|3212683	Chain F, Enoyl-Coa Hydratase Complexed With Octanoyl-Coa	256	28269.5	6.41	SLAMEMVLTGDR/NSSVGLIQLNRPK	$P < 0.05$
5	gi|72679567	Similar to Nit protein 2 (rat)	102	30681.7	6.9	LALIQLQVSSIK	$P < 0.05$
6	gi|51261047	Homolog of ES1 (rat)	86	28154.8	9.11	ITNLAQLSAANHDAAIFPG	$P < 0.05$
7	gi|27676450	PREDICTED: similar to HSCO protein (rat)	63	27659	6.58	LSGAQADLHIGEGDSIPF	$P < 0.05$
8	gi|38181885	Suclg1 protein (rat)	151	34995.4	9.54	HLGLPVFNTVK/AKPVVSFIAGITAPPGR	$P < 0.05$
9	gi|266685	Dihydro lipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (E2)	67	58726.9	5.7	VPEANSSWMDTVIR	$P < 0.05$
10	gi|1352051	ATP synthase D chain, mitochondrial	83	18751.6	6.17	LASLSEKPPAIDWAYYR	$P < 0.05$
11	gi|62531217	Ndufa10 protein (rat)	169	40467.6	7.64		$P < 0.05$
12	gi|1352624	Pyruvate dehydrogenase E1 component beta beta subunit, mitochondrial precursor (PDHE1-B)	106	38822.8	5.94	IMEGPAFNFLDAAVR	$P < 0.05$
13	gi|34865350	PREDICTED: branched chain keto acid dehydrogenase E1, beta polypeptide (rat)	78	42795.8	6.41		$P < 0.05$
14	gi|57657	Pyruvate dehydrogenase E1 alpha form 1 subunit (rat)	78	43168.6	8.35		$P < 0.05$
15	gi|38303969	Heat shock 70 kDa protein 5 (rat)	258	72302.4	5.07	VTHAVVTVPAYFNDAQR/DNHLLGTFDLTGIPPAPR	$P < 0.05$
16	gi|13937353	Prohibitin (rat)	344	29801.9	5.57	FDAGELITQR/DLQNVNIT LR/KLEAAEDIAYQLSR	$P < 0.05$
17	gi|205628	24-kDa mitochondrial NADH dehydrogenase precursor	221	26511.4	6	DIEEIIDELR/AAAVLLAQR/DTPENNPDTPFDFTPENY	$P < 0.05$
18	gi|59808474	Suclg2 protein	96	41694.8	5.64	LEGTNVQEAQNILK/EQIDIFEGIKDSQAQR	$P < 0.05$
19	gi|62662511	PREDICTED: similar to glyceraldehyde-3-phosphate dehydrogenase (rat)	78	35800.1	7.63		$P < 0.05$
20	gi|37590767	GAPD protein (rat)	102	35805.2	8.14		$P < 0.05$
21	Not identified						$P < 0.05$
22	Not identified						$P < 0.05$
23	gi|62655085	PREDICTED: hypothetical protein XP_579392 (rat)	78	115062.3	6.37	GQILTMANPIIGNGGAPD	$P < 0.05$
24	gi|47938958	Mitochondrial acyl-CoA thioesterase 1 (rat)	67	49597.7	8.16	GLAPEQPVTLR	$P < 0.05$
25	Not identified						$P < 0.05$

The proteins were identified by PMF and MS/MS using the program MASCOT v. 1.9 against SWISS-PROT database with GPS explorer software. Protein scores were based on combined MS and MS/MS spectra. Many proteins were identified by combined mode, but a few proteins were only matched by PMF, maybe because MS/MS data was poor. Only protein identifications with score greater than $P < 0.05$ were considered to be positive. No: the spot labels, whose position on the 2-DE gels were displayed in Figure 1B.
Note: Statistical analysis of the density of differential spots in the 2-DE gels. Experiment was performed in triplicate. The results were applied to *t*-test analysis.

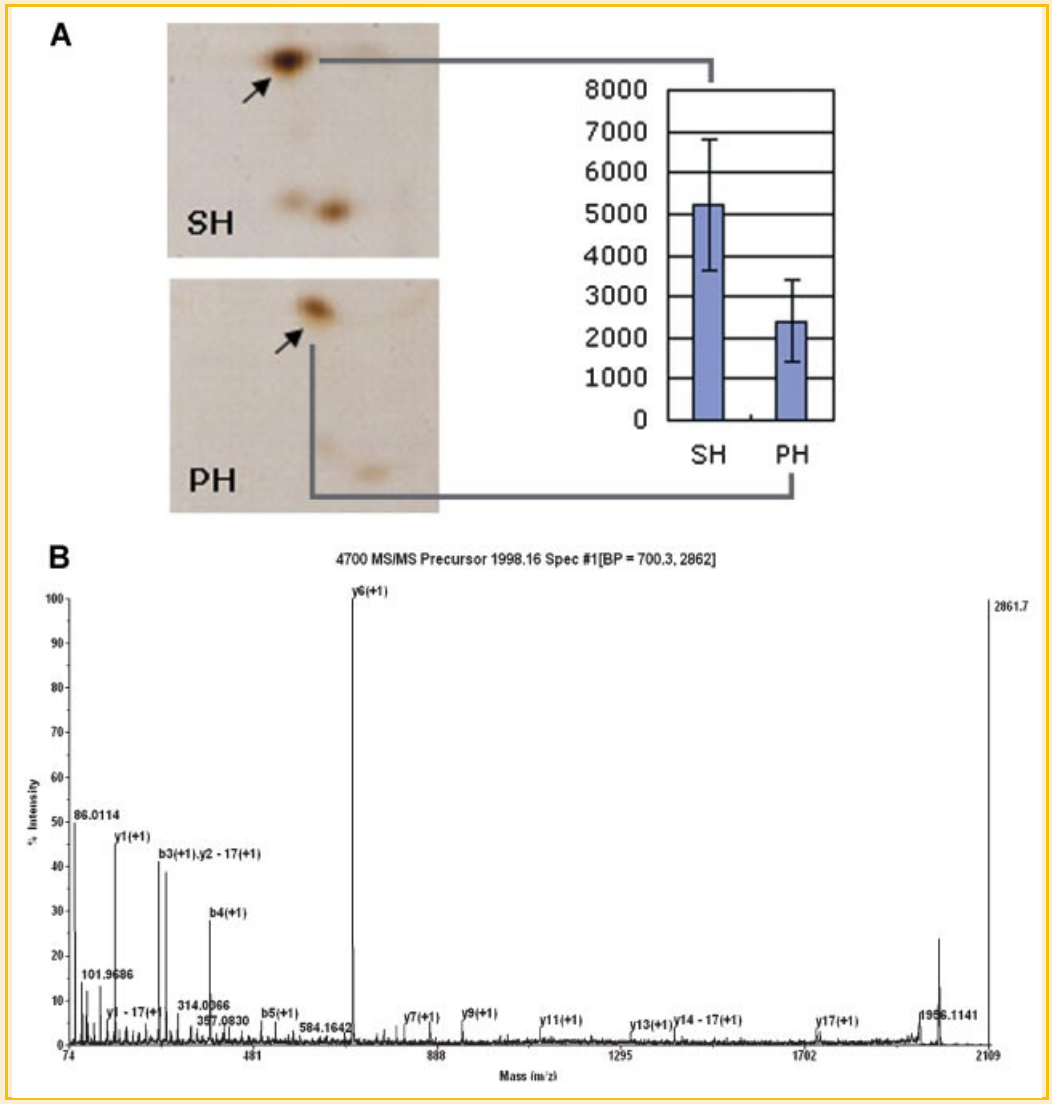

Fig. 2. Prohibitin (Fig. 1B, spot 16) was down-regulated at 24 h after 70%PHx. A: Close-up sections comparing the intensities of the prohibitin in SH and PH groups. Experiment was performed in triplicate and bar graphs represent mean ± SD of density derived from PDQuest7.4 software. B: MALDI-MS/MS sequencing result of the peptide 1998.16. The peptide sequence was identified as AAELIANSLATAGDGLIELR, which led to the identification of prohibitin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

response to PHx have been seen mainly as providing a useful form of energy to sustain LR [Tsai et al., 1992; Inomoto et al., 1994; Guerrierie et al., 1995]. To our knowledge, this is the first description of mitochondrial proteome alterations after surgical reduction of liver volume that have been identified by 2-DE in combination with MS, and protein identification via PMF and MS/MS. A single mitochondrion in eukaryotes contains about 1,000 proteins and only 13 of these are encoded by mitochondrial DNA. Interestingly, all of the differentially expressed proteins obtained in the present study are nucleus-encoded, suggesting that the differential expression of mitochondrial proteins induced by PHx might be due to alterations in the expression of nuclear genes or their regulatory mechanism. It was reported that PHx also caused disturbances in intracellular homeostasis such as overproduction of reactive oxygen species (ROS) [Yang et al., 2004] and Ca^{2+} overloading [Kanno et al., 2004] in the early phase of LR. ROS could attack nuclear DNA directly and cause hydrogen bond breaks, base pair degradation as well as double helix unwinding. Excessive activation of Ca^{2+}-dependent protease can also lead to the rupture of DNA structure. Thus, nuclear DNA was injured by PHx-induced biological effects in multisteps, and the transcription as well as expression of nucleus encoded proteins was changed. This not only influenced the expression and function of proteins in cells, but also disturbed protein distribution patterns.

In our study, we have identified 22 differentially expressed proteins (Table I). Bioinformatics analysis suggested that they might regulate many of the biological processes in the remnant livers after PHx, including carbohydrate metabolism, lipid metabolism, the respiratory chain and oxidation–phosphorylation, biotransformation and other metabolic pathways. It is reasonable to believe that

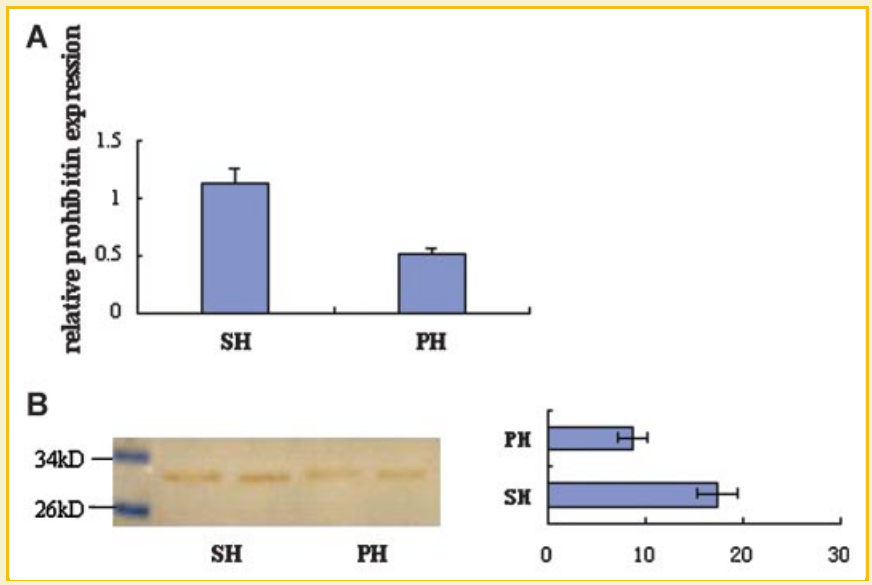

Fig. 3. Validation of differential protein by quantitative real-time PCR and Western blotting. A: Altered expression of prohibitin mRNA at 24 h after 70%PHx. Rat livers were homogenized and harvested with Trizol reagent. Real-time PCR amplifications were performed, standardized by the amounts of β-actin. The real-time PCR signals were analyzed with LightCycler 3.5 software. B: Western blotting for one of the differential proteins, prohibitin. Samples of mitochondrial protein were analyzed on 10% SDS–PAGE, followed by immunoblotting and visualized with DAB. On the left are the Western blotting results for prohibitin and on the right is a gray analysis of the results. SH: sham-operation control groups; PH: 70%PHx groups. All experiments were performed in triplicate and the data were represented as means ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

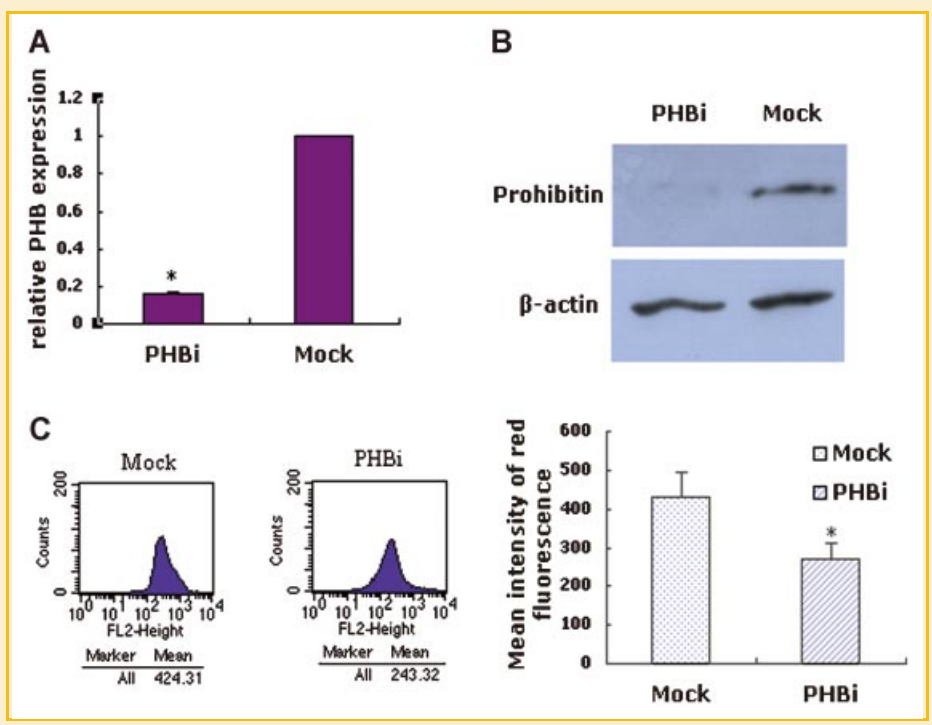

Fig. 4. PHBi induced the loss of mitochondrial membrane potential. A: BRL-3A cells were seeded in 6-well plates and transfected with 100 nM siRNA duplex targeting prohibitin and nonsensing duplex. At 48 h post-transfection, cells were harvested with Trizol reagent. Real-time PCR amplifications were performed and the mRNA expression of prohibitin were standardized by the amounts of β-actin. The real-time PCR signals were analyzed with LightCycler 3.5 software. B: Or after 72 h post-transfection, cells were lysed with SDS–PAGE loading buffer. Proteins were analyzed on 10% SDS–PAGE, followed by immunoblotting with antibodies against prohibitin and β-actin. C: Decreased of mitochondrial membrane potential in PHBi cells. FACS analysis was performed with PHBi and mock (cells transfected with nonsensing duplex, used as control). FL2 exhibits red fluorescence showing depolarization of mitochondrial potential. All experiments were performed in triplicate and the data were represented as means ± SD. A standard *t*-test was used and *P* values were generated to establish the significance level of the data and comparisons between PHBi and mock; $^{*}P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

these proteins might also be involved in the regulation of LR through different pathways and in different manners. Several identified proteins are related to energy metabolism, such as the dihydro lipoyllysine residue acetyltransferase component of pyruvate dehydrogenase complex (E2), the pyruvate dehydrogenase E1 β-subunit mitochondrial precursor (PDHE1-B), the branched chain keto acid dehydrogenase E1 β polypeptide and pyruvate dehydrogenase E1 α form 1 subunit. Most of them take part in the Krebs cycle, which is responsible for converting pyruvic acid into hydrogen, carbon dioxide and electrons, finally producing ATP. Significantly, the other down-regulated proteins identified were enzymes involved in lipid metabolism, including acyl-coenzyme A dehydrogenase (ACDM), the short chain and chain F of enoyl-CoA hydratase (ECHM) complexed with octanoyl-CoA. These proteins participate in the process of fatty acids β-oxidation, and ACDM might be involved in rate limiting and regulation [Broadway et al., 1992; Aoyama et al., 1994]. A disorder in lipid metabolism might be an important characteristic of the PHx-induced dysfunction of mitochondria. The respiratory chain-related proteins detected in this study, such as the ATP synthase D chain and the 24 kDa mitochondrial NADH dehydrogenase precursor, were also down-regulated and are involved in mitochondrial electron transport. Some proteins known to be related to biotransformation, such as mitochondrial aldehyde dehydrogenase (ALDH) precursor and preornithine carbamoyl transferase, were also identified in this study. There are reports that mitochondria showed a decrease in the rate of ATP synthesis, oxidative phosphorylation and a diminution of the respiratory control index, whereas the energy charge in the early phase of LR was restored to normal levels within 7 days [Inomoto et al., 1994; Guerrierie et al., 1995; Steer, 1995]. In agreement with an earlier report, our results suggested a dysfunction of mitochondria at 24 h after 70%PHx, which may be involved in causing the lag of LR at this phase [Guerrierie et al., 1995]. It is a plausible, albeit incomplete, suggestion from others and our data that mitochondria might have a preparing role in the early phase of LR, and thereafter it has an important role in remodeling of the liver tissue.

It is interesting to observe that prohibitin (Fig. 2) was down-regulated in mitochondria at 24 h after 70%PHx. It had been proposed that prohibitin was involved in many cellular processes, such as cell cycle regulation, transcription regulation, tumor suppression and apoptosis [Roskams et al., 1993; Welburn and Murphy, 1998; Wang et al., 1999]. LR is a very complicated biological procedure which involves various signal transduction pathways and molecular events. Thus, we thought that prohibitin may have a crucial role during LR and chosen this protein for further investigations. Prohibitin was reported to mainly localize in mitochondria, with homologues from yeast to man. Though a growing body of evidence has implicated a role of prohibitin in mitochondrial structure, function and inheritance [Nijtmans et al., 2000, 2002; Thompson et al., 2003], the role of prohibitin in mitochondria have not been completely elucidated and little is known about the molecular function of prohibitin during LR. The mitochondrial-localized prohibitin is confirmed as a high-molecular-weight hetero-complex (ring-shaped structure) by single particle structures [Tatsuta et al., 2005]. Moreover, the interaction of nonassembled respiratory chain subunits with the prohibitin complex has lead to the proposal of a chaperone activity of prohibitins during the biogenesis of the respiratory chain [Nijtmans et al., 2000]. In support of this observation, the down-regulated mitochondrial respiratory enzymes found in this study might be related to the decreased expression of prohibitin. By applying RNAi (PHBi) with BRL-3A, prohibitin knock-down demonstrated a decreased mitochondrial membrane potential (Fig. 4C), implying a role in maintaining mitochondrial integrity. An earlier report suggested that prohibitin acts as an E2F repressor and as a blocker in some earlier events, such as disruption of mitochondrial integrity and release of cytochrome *c* [Fusaro et al., 2002]. It was reported recently that prohibitin protects cells from anthralin-induced death by maintaining mitochondrial integrity [Kim et al., 2007]. And some recent study discovered that overexpression of prohibitin inhibits the collapse of the electrochemical gradient across the mitochondrial membrane during serum withdrawal-induced apoptosis in granulosa cells [Chowdhury et al., 2007].

In summary, the analysis of altered proteome of mitochondria proved to be an effective approach for better understanding the role of mitochondria during LR. The changes in the expression of several proteins after 70%PHx reflect the involvement of various cellular metabolic pathways. Our results identified several cellular functions, not yet studied in the context of LR, as valuable targets for further investigation. Prohibitin was shown to be involved in the maintenance of mitochondrial integrity, which might provide an approach to understanding the mechanism underlying the changes of mitochondria in the early phase of LR.

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