Comparative Studies of Early Liver Dysfunction in Senescence-accelerated Mouse Using Mitochondrial Proteomics Approaches* \[8\]

Yashu Liu‡§, Jintang He‡§, Shaoyi Ji¶, Qingsong Wang‡, Hai Pu‡, Tingting Jiang‡, Lingyao Meng‡, Xiuwei Yang¶, and Jianguo Ji‡

The liver is a complex and unique organ responsible for a breadth of functions crucial to sustaining life, especially for various metabolic processes in its mitochondria. Senescence-accelerated mouse prone/8 (SAMP8), a widely used aging model, exhibits an oxidative stress-induced aging phenotype and severe mitochondria-related liver pathology that are not seen in senescence-accelerated mouse resistant/1 (SAMR1). Here we used both two-dimensional electrophoresis- and ICAT-based mitochondrial proteomics analysis to view the liver mitochondrial protein alterations between SAMP8 and SAMR1. Compared with SAMR1, decreased expression and activity of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase were detected in SAMP8 at 6 months old (SAMP8-6m). As the key enzyme of ketogenesis, 3-hydroxy-3-methylglutaryl-CoA synthase is well known to be transcriptionally regulated by peroxisome proliferator-activated receptor α, which was also expressed at lower levels in SAMP8-6m livers. In addition, down-regulation of two peroxisome proliferator-activated receptor α target gene products (acyl-CoA oxidase and enoyl-CoA hydratase), elevation of triglyceride, and reduction of acetyl-CoA were observed, indicating abnormal fatty acid metabolism in SAMP8-6m livers. In addition eight proteins (NDUAA, NDUBA, NDUB7, NDUS1, NDUS3, NDUV1, ETFα, and UCRI) of mitochondrial complexes were down-regulated in SAMP8-6m, resulting in mitochondria-related liver dysfunction characterized by enhanced oxidative stress-induced molecular damage (lipid peroxide and oxidized protein) and depressed energy production (ATP). Glutamine synthetase and ornithine aminotransferase involved in glutamine synthesis were up-regulated in SAMP8 livers at both 1 and 6 months old that may be related to the accumulation of glutamate and glutamine. Our work provided useful clues to understanding the molecular mechanism underlying liver dysfunction in senescence-accelerated mouse. Molecular & Cellular Proteomics 7:1737–1747, 2008.

The liver is regarded as the major organ for a number of physiological presses. As the main energy producers, liver mitochondria are considered to be the central integrators of intermediary metabolism including fatty acid oxidation, the Krebs cycle, oxidative phosphorylation, ketogenesis, and the urea cycle. Furthermore most of the liver disorders have been reported to have increased reactive oxygen species (ROS)\[1\] and decreased ATP as vital characteristics (1, 2), which are both generated through the mitochondrial respiratory chain.

The senescence-accelerated mouse (SAM) model is an aging model obtained by continuous sister-brother breeding from original litters of AKR/J mice (3). SAM includes two strains, i.e. SAM prone (SAMP) and SAM resistant (SAMR). SAM exhibit a shortened life span and early manifestation of various symptoms of senescence, whereas SAMR are senescence-resistant inbred strains (4). SAMP8, a substrain of SAMP, has become a major biogerontological resource in aging research with SAMR1 as control (5, 6).

Recently Ye et al. (7) have suggested SAMP8 as a valuable animal model for the study of liver diseases because of its phenotype of liver dysfunction. Compared with SAMR1, 5-month-old SAMP8 display hepatic steatosis and reduced fatty acid oxidation (8). The levels of alanine aminotransferase and aspartate aminotransferase were also significantly increased in SAMP8, indicating abnormal liver functions. Oxidative stress, as a crucial component of most liver pathologies (9, 10), was found to be elevated in aged SAMP8 mice livers, supporting the relationship between liver pathologies and free radical damage induced by mitochondrial impairment (11, 12). Although various functional

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* The abbreviations used are: ROS, reactive oxygen species; 2-DE, two-dimensional gel electrophoresis; SAM, senescence-accelerated mouse; SAMP, senescence-accelerated mouse prone; SAMP8-1m, senescence-accelerated mouse prone/8 at 1 month old; SAMP8-6m, senescence-accelerated mouse prone/8 at 6 months old; SAMR, senescence-accelerated mouse resistant; SAMR1-1m, senescence-accelerated mouse resistant/1 at 1 month old; SAMR1-6m, senescence-accelerated mouse resistant/1 at 6 months old; TG, triglyceride; PPARα, peroxisome proliferator-activated receptor α; S/N, signal to noise ratio; HMG, 3-hydroxy-3-methylglutaryl; 2-D, two-dimensional; ID, identity; OAT, ornithine aminotransferase; GS, glutamine synthetase.
changes have been observed in liver mitochondria of SAMP8, the molecular mechanism of these variations remains unknown.

Mitochondrial proteomics combines classic biochemical fractionation methods and robust mass spectrometry-based proteomics methods, providing insights into the identity and functions of mitochondria (13–16). Here we applied both 2-DE- and ICAT-based mitochondrial proteomics approaches to compare the different expression patterns of liver mitochondrial proteins between SAMP8 and SAMR1 at 1 and 6 months old. We found that most of the down-regulated proteins in SAMP8 were related to fatty acid metabolism, the tricarboxylic acid cycle, and oxidative phosphorylation, whereas the up-regulated proteins were mainly involved in glutamine synthesis. Consistent with the protein alteration, we also found that triglyceride (TG), glutamine, oxidative protein, and lipid peroxide concentrations were increased in 6-month-old SAMP8 livers, whereas acetyl-CoA and ATP content were decreased. Our results provided useful information to understanding the early stage of liver dysfunction in senescence-accelerated mouse at the proteome level.

MATERIALS AND METHODS

Isolation of Livers—Male SAMP8 at 1 month old (SAMP8-1m), SAMP8 at 6 months old (SAMP8-6m), SAMR1 at 1 month old (SAMR1-1m), and SAMR1 at 6 months old (SAMR1-6m) (obtained from the Institute of Genetics and Developmental Biology of the Chinese Academy of Sciences) were housed under specific pathogen-free conditions at a temperature of 25 ± 1.5°C with 12:12-h light-dark cycles with full access to water and food. Animals were anesthetized intraperitoneally with 10% (w/v) chloral hydrate and tissues were removed and homogenized immediately for the following procedures.

Preparation of Liver Mitochondria—Mitochondria were isolated by subcellular fractionation from fresh livers as described previously (17) with some modifications. Briefly minced livers were homogenized with 5 volumes of ice-cold homogenization buffer (0.25 M sucrose, 10 mM HEPES, 1 mM PMSF, pH 7.5) using a loose fitting Dounce homogenizer. After filtering through four layers of nylon gauze, the supernatant was centrifuged at 15,000 g for 20 min in a Himac 1.5°C with 12:12-h system (Micro-Tech Scientific, Vista, CA). The ICAT labeling was performed according to the manufacturer’s procedures. Samples from six SAMP8 mice at 1 month old were labeled with the heavy reagent, six SAMR1 mice at 1 month old were labeled with the light reagent (forward labeling), and then a reverse labeling with samples obtained from an independent experiment was also performed to avoid variability of the labeling procedure. The same labeling methods were applied to samples from SAMP8 and SAMR1 mice at 6 months old.

Nano-LC-FTICR-MS—The ICAT-labeled peptides were dissolved in 0.1% formic acid and then separated by a nano-LC system (Micro-Tech Scientific, Vista, CA) that was equipped with a strong cation exchange column and a C18 reverse phase column. The salt steps were 5, 10, 20, 50, 100, and 500 mM NH₄Cl, each of which was followed by a 120-min reverse phase gradient from 0 to 50% acetonitrile in 0.1% formic acid at a constant flow rate of 400 nL/min. Mass spectra were recorded on a 7-tesla FTICR mass spectrometer, Apex-Qe (Bruker Daltonics, Bremen, Germany). Data were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics). The strongest peak of each MS spectrum was selected for the following MS/MS analysis. The MS/MS spectra were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics). The strongest peak of each MS spectrum was selected for the following MS/MS analysis. The MS/MS spectra were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics). The strongest peak of each MS spectrum was selected for the following MS/MS analysis. The MS/MS spectra were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics). The strongest peak of each MS spectrum was selected for the following MS/MS analysis. The MS/MS spectra were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics). The strongest peak of each MS spectrum was selected for the following MS/MS analysis. The MS/MS spectra were acquired in data-dependent mode using ApexControl 1.0 software (Bruker Daltonics). The strongest peak of each MS spectrum was selected for the following MS/MS analysis.
S/N ≥ 4.0 and automatically searched against the Swiss-Prot database (version 54.0) using Mascot 2.1.0 (Matrix Science). Mass tolerances for MS and MS/MS were 30 ppm and 0.03 Da, respectively, and variable modifications were specified as ICAT_heavy and ICAT_light. The instrument setting for the Mascot search was selected as “ESI-FTICR.” Finally the ratio of the detected peptide pairs was obtained by calculating their extracted ion chromatograms with WarPLC 1.1 software and Biotool 3.1 (Bruker Daltonics). Furthermore only precursor ions with peak intensity ≥10⁶ and S/N ≥ 20 were accepted for quantitation.

Quantitative Real Time PCR—Primers are listed in Table III. The reverse transcription was performed on 2 μg of total RNA with SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real time PCR was performed using the SYBR Green PCR kit (Applied Biosystems) in a DNA Engine Opticon-Continuous Fluorescence Detection System (MJ Research) with parameters as follows: initiation with a 10-min denaturation at 95 °C followed by 40 cycles of amplification with 10-s denaturation at 94 °C, 20-s annealing of the primer pair at 58 °C, 30-s extension at 72 °C, and reading the plate for fluorescence data collection at 78–80 °C. After a final extension at 72 °C for 5–10 min, a melting curve was performed from 65 to 95 °C (1-s hold/0.2 °C increase) to check the specificity of the amplified product. Mouse β-actin was used as an internal control in parallel reactions with primers. All samples were analyzed in triplicates using independent RNA samples.

Assay of 3-Hydroxy-3-methylglutaryl (HMG)-CoA Synthase Activity—HMG-CoA synthase activity was assayed as described by Quant et al. (22). Briefly 0.2 g of livers was homogenized in 1 ml of homogenization buffer (0.25 m sucrose, 10 mM HEPES, 1 mM PMSF, pH 7.5), then 100 μl of homogenates were treated with 5 μl of 20% Triton X-100, and 5-μl samples were assayed immediately. The 1-ml assay system contained 50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 0.2 mM DTT at 30 °C; 5 mM acetyl phosphate, 10 μM acetoacetyl-CoA, and 5-μl samples were added followed by addition of 10 units of phospho-transacetylase and 100 μM acetyl-CoA. HMG-CoA synthase activity was measured as the difference in the rate before and after addition of acetyl-CoA. A unit of enzyme activity is defined as 1 μmol of acetyl-CoA formed/min.

Glutamine Synthetase Activity and Glutamine Assays—Glutamine synthetase activity was assayed by measuring the rate of formation of γ-glutamyl hydroxamate as described previously (23). By definition, 1 unit of enzyme activity causes 1 μmol of γ-glutamyl hydroxamate formed/min. Glutamine concentration was determined with a glutamine/glutamate determination kit (Sigma-Aldrich) following the manufacturer’s instructions.

Detection of the Concentration of Protein Carbonyl and Lipid Peroxide—The level of protein carbonyl was determined according to the manufacturer’s instructions. The level of protein carbonyl was determined according to the method of Oliver et al. (24). Carbonyl groups in oxidized proteins were derivatized to 2,4-dinitrophenylhydrazone, and then absorption at 360 nm was measured. Lipid peroxide in liver was measured by the thiobarbituric acid reaction as described previously (25).

Triglyceride, Acetyl-CoA, and ATP Determination—Triglyceride, acetyl-CoA, and ATP were measured in liver homogenates as described by Roglans et al. (26), Baltazar et al. (27), and Yu et al. (28), respectively.

RESULTS

Evaluation of the Isolated Mitochondria—The purity of the mitochondria was examined by Western blotting. We compared the liver homogenates with the isolated mitochondria using antibodies against Oxphos complex I 39-kDa subunit. As a result, the mitochondria-specific protein was enriched about 7-fold in the mitochondria fraction (Fig. 1A). The mitochondria fraction was also morphologically evaluated by electron microscopy. As can be seen in Fig. 1B, the predominant structures in this fraction were mitochondria.

2-DE-based Proteomics Analysis—The proteins of mouse liver mitochondria were separated by 2-DE. Four representative 2-D gel images (Fig. 2) were divided into two groups, i.e. a 1-month group (Fig. 2, A and B) and a 6-month group (Fig. 2, C and D). Comparisons among these images resulted in the detection of 10 differentially expressed proteins between SAMP8 and SAMR1 in each group that were further analyzed by MALDI-TOF MS and MALDI-TOF/TOF MS after tryptic in-gel digestion. The combined peptide mass fingerprint and LIFT MS/MS spectra were searched against the Swiss-Prot database using a Mascot engine (MALDI-TOF/TOF mass spectra of peptides from the changed proteins are shown in supplemental Fig. S1, and the detailed information of Mascot search results is listed in supplemental Table S1). Table I lists the identified protein IDs together with their spot numbers, molecular weights and pI values, amount of peptides, -fold changes, subcellular locations, and functions. The higher magnification images of one protein, HMG-CoA synthase, are shown in Fig. 5A.

ICAT-based Proteomics Analysis—ICAT technology was applied to quantitatively assess the overall protein expression ratios between SAMP8 and SAMR1. Both forward and reverse labeling experiments were applied to samples from SAMR1 and SAMP8 at each month. The MS spectra were searched against Mascot 2.1.0 with mass accuracies of 30 ppm for the parent ion and 0.03 Da for fragment ions. The ion cutoff score
for accepting individual MS/MS spectra was 14, resulting in a false-positive rate of 1% as evaluated by a composite target/decoy Swiss-Prot database. One hundred and forty-eight proteins were identified from SAMR1-1m and SAMP8-1m in both forward and reverse labeling experiments (see supplemental Table S2), and 166 proteins were identified from the 6-month group (see supplemental Table S3). 51% (99 of 196) of these proteins were located to mitochondria (see supplemental Table S4).

A linear regression analysis of the SAMP8/SAMR1 ratios from experiment 1 (SAMR1 labeled with the light chain) and experiment 2 (SAMP8 labeled with the light chain) was conducted (Fig. 3). The Pearson correlation coefficient was 0.75 in the 1-month group and 0.83 in the 6-month group with p value <0.0001.

The variation in the SAMP8/SAMR1 ratios is displayed in Fig. 4. Proteins were categorized into different bins according to their ratio with 0.125 unit/bin. More than 90% of the proteins had expression ratios between 0.7 and 1.45 in both 1- and 6-month groups. Therefore, 13 proteins with a ratio of ≥1.5 or ≤0.67 were considered to be significantly changed (Table II; the spectrum for each peptide is illustrated in supplemental Fig. S2) wherein HMG-CoA synthase and ornithine aminotransferase were also identified from 2-D gels. The MS spectra of two ICAT-labeled peptides from HMG-CoA synthase are shown in Fig. 5B.

Quantitative Real Time RT-PCR—Eight altered proteins were selected to analyze their mRNA expression differences. The mRNA expression variations were in excellent agreement with the changing patterns of proteins (Table III). As an example, Fig. 5C shows the change of mRNA expression of HMG-CoA synthase.

HMG-CoA Synthase Activity—The activity of HMG-CoA synthase in liver homogenates was measured. For this enzyme activity, there was not a significant difference between SAMR1-1m and SAMP8-1m. However, compared with SAMR1-6m, the HMG-CoA synthase activity in SAMP8-6m was reduced about 33.3% (p < 0.05) (Fig. 5D).

Expression Levels of PPARα—It is well known that transcription activation of HMG-CoA synthase is mainly influenced by PPARα. Therefore, we analyzed the expression levels of PPARα. Western blotting analysis showed that
PPARα was clearly down-regulated in SAMP8-6m livers compared with SAMR1-6m, whereas the expression levels of the protein were almost identical in 1-month-old mice livers (Fig. 6A). A similar expression pattern of PPARα at the level of mRNA was also found (Fig. 6B).

Assays of Glutamine Synthetase and Ornithine Aminotransferase—Glutamine synthetase and ornithine aminotransferase, which are related to glutamine synthesis, were identified as up-regulated proteins in SAMP8 using proteomics approaches. This was further verified by Western blotting analysis. Consistent with the proteomics results, both of these proteins were clearly more abundant in SAMP8 liver homo-

### TABLE I

| No. | Swiss-Prot ID | Protein name                        | Molecular weight/pl | Scorec | Pep. | Ratio 1m⁴ | Ratio 6m⁴ | Loc. | Function                  |
|-----|--------------|-------------------------------------|---------------------|--------|------|----------|----------|------|--------------------------|
| 1   | OAT_MOUSE    | Ornithine aminotransferase          | 48,723/6.19         | 336    | 4    | 1.56     | 2.72     | MC   | Transaminase              |
| 2   | HACL1_MOUSE  | 2-Hydroxyacyl-CoA lyase 1           | 64,588/5.89         | 532    | 4    | 1.23     | 0.68     | Pero | Lipid metabolism         |
| 3   | HMCS2_MOUSE  | HMG-CoA synthase                    | 57,300/8.65         | 115    | 1    | 1.1      | 0.63     | MC   | Ketone body biosynthesis  |
| 4   | ODO2_MOUSE   | Dihydrolipoamide succinyltransferase| 49,306/9.11         | 194    | 2    | 0.96     | 0.45     | MC   | Tricarboxylic acid cycle  |
| 5   | NDUBA_MOUSE  | NADH-ubiquinone oxidoreductase      | 21,296/8.19         | 342    | 3    | 1.06     | 0.62     | MC   | Respiratory chain         |
| 6   | NDUA2_MOUSE  | NADH-ubiquinone oxidoreductase      | 40,863/7.63         | 338    | 4    | 0.93     | 0.48     | MC   | Ketone body biosynthesis  |
| 7   | NDUS3_MOUSE  | NADH-ubiquinone oxidoreductase      | 30,302/6.67         | 530    | 6    | 0.89     | 0.5      | MC   | Respiratory chain         |
| 8   | UCRI_MOUSE   | Ubiquinol-cytochrome c reductase    | 29,634/8.91         | 196    | 2    | 0.9      | 0.67     | MC   | Respiratory chain         |
| 9   | ETFA_MOUSE   | Electron transfer flavoprotein      | 35,330/8.62         | 404    | 3    | 1.05     | 0.61     | MC   | Respiratory chain         |
| 10  | PRDX3_MOUSE  | Thioredoxin-dependent peroxide      | 28,337/7.15         | 155    | 2    | 1.43     | 1.85     | MC   | Oxidative damage protection |

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**FIG. 3.** Scatter plots obtained from orthogonal labeling in 1-month-old mice (A) and 6-month-old mice (B). The SAMP8/SAMR1 ratio of each identified protein from Experiment 1 (Exp1) or Experiment 3 (Exp3) (SAMR1 labeled with the light chain) is plotted on the x axis, and that from Experiment 2 (Exp2) or Experiment 4 (Exp4) (SAMP8 labeled with the light chain) is plotted on the y axis. The dashed line indicates a linear regression with 95% confidence.

**FIG. 4.** Variation of identified ICAT-labeled peptides versus SAMP8/SAMR1 ratio in 1-month-old mice (A) and 6-month-old mice (B).

*Protein numbers on the 2-D gel (see Fig. 2).  
Molecular weight search (MOWSE) scores obtained by the combined search (peptide mass fingerprint and LIFT data) using Mascot engine.  
Numbers of peptides identified by MALDI-TOF/TOF MS/MS using the Ultraflex instrument in LIFT mode.  
Protein expression ratios of SAMP8/SAMR1 at 1 month old.  
Protein expression ratios of SAMP8/SAMR1 at 6 months old.  
Protein subcellular locations. MC represents mitochondria, and Pero represents peroxisome.
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Table II

Changed proteins identified by ESI-FTICR-MS

| No. | Swiss-Prot ID | Protein name | Molecular weight | Score | Pep. | Ratio 1m/6m | Loc. | Function |
|-----|---------------|--------------|------------------|-------|------|------------|------|----------|
| 1   | OAT_MOUSE     | Ornithine aminotransferase | 48,324 | 14  | 1    | 1.55      | 2.3  | MC       |
| 2   | GLNA_MOUSE    | Glutamine synthetase | 42,092 | 100 | 2    | 1.82      | 2.1  | MC       |
| 3   | AL4A1_MOUSE   | Δ^1-Pyrroline-5-carboxylate dehydrase | 61,772 | 38  | 2    | 1.5       | 1.03 | Cyto     |
| 4   | URIC_MOUSE    | Uricase | 35,017 | 65  | 2    | 0.88      | 0.43 | Cyto     |
| 5   | ACOX1_MOUSE   | Acyl-coenzyme A oxidase 1 | 74,587 | 146 | 3    | 1.02      | 0.64 | MC and Pero |
| 6   | ECHP_MOUSE    | Enoyl-CoA hydratase | 78,194 | 132 | 4    | 1.05      | 0.57 | MC and Pero |
| 7   | HMCS2_MOUSE   | HMG-CoA synthase | 56,786 | 109 | 3    | 1.15      | 0.65 | MC       |
| 8   | NDUS1_MOUSE   | NADH-ubiquinone oxidoreductase 75-kDa subunit | 79,698 | 39  | 1    | 0.72      | 0.5  | MC       |
| 9   | NDUB7_MOUSE   | NADH-ubiquinone oxidoreductase B18 subunit | 16,320 | 62  | 1    | 0.95      | 0.63 | MC       |
| 10  | NDUV1_MOUSE   | NADH-ubiquinone oxidoreductase 51-kDa subunit | 50,802 | 50  | 2    | 0.89      | 0.62 | MC       |
| 11  | BHM1_MOUSE    | Betaine-homocysteine S-methyltransferase 1 | 44,992 | 236 | 4    | 2.2       | 1.05 | Cyto     |
| 12  | CAH3_MOUSE    | Carbonic anhydrase 3 | 29,348 | 21  | 1    | 0.31      | 0.32 | Cyto     |
| 13  | HYEP_MOUSE    | Epoxide hydrolase | 52,542 | 53  | 1    | 1.0       | 0.58 | ER       |

a Molecular weight search (MOWSE) scores obtained by the search of MS/MS data using Mascot engine.
b Numbers of peptides identified by FT-MS.
c Protein expression ratios of SAMP8/SAMR1 at 1 month old.
d Protein expression ratios of SAMP8/SAMR1 at 6 months old.
e Protein subcellular locations. MC represents mitochondria, Pero represents peroxisome, Cyto represents cytoplasm, and ER represents endoplasmic reticulum.

In addition, the activity of glutamine synthetase was measured, and it was significantly increased in livers of both SAMP8-1m and SAMP8-6m (Fig. 7A). The liver glutamine concentration was also determined. As shown in Fig. 7C, glutamine concentration was slightly increased in SAMP8 but only statistically significant in 6-month-old SAMP8.

Oxidation Levels of Liver Proteins and Lipids—Both protein and lipid oxidation levels were compared between SAMR1 and SAMP8 at 1 and 6 months old. Protein carbonyl content (p < 0.01) and lipid peroxide (p < 0.05) in SAMP8-6m were significantly increased (Fig. 8, A and B).

Other Assays—Table IV lists several attributes of SAMR1 and SAMP8. Body weights and liver weights had no statistical differences between SAMR1 and SAMP8. As compared with SAMP8-6m, the liver triglycerides in SAMP8-6m were significantly increased (p < 0.01), whereas the levels of liver ATP (p < 0.01) and of liver acetyl-CoA (p < 0.05) were significantly decreased. Note that none of these characteristics had significant differences between SAMR1-1m and SAMP8-1m.

**DISCUSSION**

As a widely used aging model, SAMP8 shows various age-related disorders after normal development and maturation. It has been reported that the differences between SAMP8 and SAMR1 become obvious after 4–6 months of age due to the steeper increase in the degree of senescence (29). In our study, no statistical difference was observed between the body weight, liver weight, and liver/body weight ratio of SAMP8 and that of SAMR1 at both 1 and 6 months old. However, SAMP8-6m showed a significant increase of liver lipid peroxide, protein carbonylation, and triglyceride concentration, indicating mitochondria-related liver dysfunction at the early stage. Therefore, we compared liver mitochondrial protein profiles between SAMP8-6m and SAMR1-6m to investigate the protein changes related to abnormal liver functions. Meanwhile protein profiles of SAMP8-1m and SAMR1-1m were also compared as a control group in the present study.

Mitochondrial metabolism of the fatty acids plays an important role in various biochemical reactions and metabolic pathways. Fatty acids constitute a main source of energy through β-oxidation with generation of acetyl-CoA subunits that may enter either the tricarboxylic acid cycle or ketogenesis in liver. Compared with SAMR1-6m, both protein and mRNA expression levels of HMG-CoA synthase were decreased in SAMP8-6m, causing significant reduction of its activity (Fig. 5). HMG-CoA synthase, which catalyzes acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA, is considered to be the control site of the ketogenesis pathway (30). Ketogenesis occurs mainly in the liver, producing ketone bodies that can be exported out of the liver to provide energy for other tissues such as the heart, skeletal muscles, and brain under certain circumstances (31). The
deficiency of HMG-CoA synthase would lead to lessened ketogenesis in SAMP8-6m livers.

It is now well recognized that the transcription activation of HMG-CoA synthase is mainly influenced by PPAR/ because of a conserved PPAR/ responsive element in its promoter region (32). Lee et al. detected an extremely low level of HMG-CoA synthase in PPAR/ knock-out mice (33). PPAR/ is also known as a fatty acid transcriptional factor that determines the capacity of hepatic fatty acid /-oxidation. Absence of PPAR/ can lead to impairment in fatty acid /-oxidation in liver mitochondria with notable elevation in TG content (34). A considerable increased TG concentration had also been found in the livers of SAMP8-6m (Table IV), and we detected PPAR expression at protein and mRNA levels, both of which showed remarkable reduction in SAMP8-6m (Fig. 6). Acetyl-CoA, as an end product of the mitochondrial /-oxidation pathway, was also found to be decreased in SAMP8-6m livers (Table IV), indicating impairment of fatty acid metabolism. In addition, down-regulated expression levels of two PPAR target proteins, acyl-CoA oxidase 1 (ACOX1) and peroxisomal enoyl-CoA hydratase (ECHP), were detected in SAMP8-6m using proteomics tools (Table II). Given the reasons above, our data support that the impaired liver fatty acid metabolism and ketogenesis in SAMP8 was closely related to PPAR deficiency.
Liver mitochondria are considered to play a key role in metabolic process not only as the major resource of chemical energy (ATP) but also as the primary site of ROS. When reducing equivalents generated by fatty acid oxidation and the tricarboxylic acid cycle are oxidized to H2O through the transfer of electrons along the mitochondria respiratory chain,

### Table III

| mRNA expression levels of some changed proteins detected by real time PCR | Gene name | GenBank™ accession no. | Primer sequence sense ( + ) antisense ( − ) | Size (bp) | Ratio 1m/6m |
|---|---|---|---|---|---|
| ODO2 | Dlst | BC006702 | (+) 5'-AGA CGT TGC CTG GAG TCT G-3' | 114 | 0.75/0.75 |
| ETFA | Etfa | BC096645.1 | (+) 5'-AGC GGT TAC TGG TCA TGC C-3' | 171 | 0.82/0.66 |
| NDUAA | Ndufa10 | BC003439.1 | (+) 5'-TGTTGACGGATTCTGAG -3' | 180 | 0.96/0.67 |
| NDUUB | Ndufb10 | BC043013.1 | (+) 5'-CTGGATGCATTGGTTG -3' | 174 | 0.75/0.56 |
| NDUUS3 | Ndufs3 | BC119269.1 | (+) 5'-ATGGCTGCTGATGCTG -3' | 183 | 1.69/0.63 |
| UCRI | Uqcrfs1 | BC019934.1 | (+) 5'-GAAGGCTGCTGACTGCT -3' | 221 | 0.79/0.43 |
| OAT | Oat | BC008119 | (+) 5'-TGGCTGCTGAGCTGCT -3' | 137 | 0.97/0.52 |
| HMCS2 | Hmgcs2 | BC024744 | (+) 5'-TGGCTGCTGAGCTGCT -3' | 135 | 0.97/0.52 |

#### FIG. 6. Analysis of PPARα

A. Western blotting analysis of PPARα. 1, 2, 3, and 4 represent liver homogenates from SAMR1-1m, SAMP8-1m, SAMR1-6m, and SAMP8-6m, respectively. B. mRNA levels of PPARα. 1, 2, 3, and 4 represent mRNA from livers of SAMR1-1m, SAMP8-1m, SAMR1-6m, and SAMP8-6m, respectively.

#### FIG. 7. Assays of glutamine synthetase and ornithine aminotransferase

A. Western blotting analysis of glutamine synthetase and ornithine aminotransferase. 1, 2, 3, and 4 represent liver homogenates from SAMR1-1m, SAMP8-1m, SAMR1-6m, and SAMP8-6m, respectively. B. Activity of GS in liver homogenates. C. Glutamine concentration in liver homogenates. *Error bars indicate S.E. from five independent experiments; *, p < 0.05; **, p < 0.01. U, units; prot, protein.

Liver mitochondria are considered to play a key role in metabolic process not only as the major resource of chemical energy (ATP) but also as the primary site of ROS. When reducing equivalents generated by fatty acid oxidation and the tricarboxylic acid cycle are oxidized to H2O through the transfer of electrons along the mitochondria respiratory chain,
ATP and ROS are produced. In the present study, we found that eight proteins involved in the mitochondrial respiratory chain were down-regulated in SAMP8-6m, including NDUAA, NDUBA, NDUB7, NDUS1, NDUV1, and NDUS3 in mitochondrial complex I; EFTA in mitochondrial complex II; and UCRI in mitochondrial complex III. All of these altered proteins showed similar variations at their mRNA expression levels, indicating lower activity of the mitochondrial respiratory chain in SAMP8-6m. Correspondingly increased molecular damage (lipid peroxidation and protein carbonylation) as well as decreased ATP concentration was detected from the livers of SAMP8-6m. All of these altered proteins involved in the respiratory chain, ketogenesis, and fatty acid oxidation and protein metabolism may lead to the respiratory chain would inhibit the lipid oxidation process (36). Therefore, it seems rational that the impairment of lipid oxidation, the tricarboxylic acid cycle, ketogenesis, and oxidative phosphorylation are closely related to each other to induce the mitochondria-related liver dysfunction in SAMP8.

As one of the main biological macromolecules, proteins are susceptible to free radical attack, leading to the loss of functions (37). Furthermore with ammonia as an end product, protein metabolic disorder may cause higher concentration of free ammonia. In our work, ornithine aminotransferase (OAT) and glutamine synthetase (GS), which can prevent ammonia accumulation via biosynthesis of glutamate and glutamine, were found to be up-regulated in both SAMP8-1m and SAMP8-6m. It has been reported previously that 2-month-old SAMP8 began to exhibit high levels of Glu and Gln, indicating abnormal protein metabolism (38). Hyperexpression of OAT from 12-month-old SAMP8 has also been reported (39); this is consistent with the present work. However, on the contrary, Butterfield et al. (40) have detected decreased GS activity in the brain of 10-month-old SAMP8 resulting from oxidative post-translational modifications. To investigate whether liver GS activity in SAMP8-6m was also influenced by oxidative modifications, we further measured the liver GS activity and glutamine (produced by GS) concentration in SAMP8-6m; both were elevated (Fig. 7, A and C), consistent with the up-regulation of GS detected by proteomics analysis (Table II) and Western blotting (Fig. 7A). However, GS expression in SAMP8 brains showed no difference from that in SAMR1, whereas its activity was significantly decreased in SAMP8-6m (supplemental Fig. S3). Considering the age-related learning and memory impairments in SAMP8, we hypothesize that the differences in these results may be due to the organ-specific control of glutamine metabolism.

Taken together, our results provided insight to explain the mitochondria-related liver dysfunction in SAMP8 at the level of subcellular proteome. We used both 2-DE- and ICAT-based proteomics analysis to find the differentially expressed mitochondrial proteins between SAMP8 and SAMR1. The altered proteins involved in the respiratory chain, ketogenesis, and fatty acid oxidation and protein metabolism may lead to

### TABLE IV

| Parameter                        | 1 month  | 6 months |
|---------------------------------|----------|----------|
|                                 | SAMR1-1m | SAMP8-1m | SAMR1-6m | SAMP8-6m |
| Number of mice                  | 5        | 5        | 5        | 5        |
| Body weight (g)                 | 23.1 ± 1.3 | 21.2 ± 1.5 | 31.3 ± 1.5 | 29.1 ± 2.1 |
| Liver weight (g)                | 1.40 ± 0.13 | 1.23 ± 0.13 | 1.71 ± 0.10 | 1.75 ± 0.17 |
| Liver weight/body weight (%)    | 6.1 ± 0.5 | 5.8 ± 0.6 | 5.5 ± 0.5 | 6.0 ± 0.3 |
| Liver triglycerides (mg/g protein) | 12.2 ± 4.9 | 14.6 ± 3.5 | 10.5 ± 3.6 | 21.7 ± 5.4* |
| Liver acetyl-CoA (nmol/mg protein) | 6.0 ± 0.8 | 5.9 ± 1.1 | 5.6 ± 0.6 | 4.1 ± 0.8* |
| Liver ATP (nmol/mg protein)     | 30.3 ± 4.1 | 27.7 ± 3.5 | 37.0 ± 3.8 | 23.4 ± 4.6* |

a p < 0.01 (Student’s t test).

b p < 0.05 (Student’s t test).
liver metabolic disorders, which are characterized by increased TG, glutamine, oxidative protein, and lipid peroxide concentration as well as decreased acetyl-CoA and ATP content in SAMPI-6m. In conclusion, our data are should be helpful in understanding the intricate process of early liver dysfunction in SAMPI-8.

* This work was supported by the National Key Basic Research Program of China (Grant 2006CB910103). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The online version of this article (available at http://www.mcponline.org) contains supplemental material.

§ Both authors contributed equally to this work.

To whom correspondence should be addressed: P. O. Box 31, College of Life Sciences, Peking University, Beijing 100871, China. Tel.: 86-10-62755470; Fax: 86-10-62751526; E-mail: jijg@pku.edu.cn.

** This work was supported by the National Key Basic Research Generation Animal Model.

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