Mice with juvenile visceral steatosis (JVS), which were originally described by Koizumi et al., display cardiac hypertrophy, fatty liver, hyperammonemia, hypoglycemia, and growth retardation; and these characteristics are inherited in an autosomal recessive manner. In JVS mice, the reabsorption system of carnitine in the kidney is defective; and, as a result, carnitine levels are low in the plasma, liver, skeletal muscle, and heart. Recently, it was proved that primary systemic carnitine deficiency in JVS mice is caused by a missense mutation in a gene encoding the sodium ion-dependent carnitine transporter. Horiuchi et al. reported that carnitine administration corrected growth retardation and abnormal gene expression of uro-cyclic enzymes and was partly effective in ameliorating the fatty liver and in suppressing the cardiac hypertrophy. These results indicate that carnitine deficiency plays an important role in the development of these pathological features. At present, JVS mice are recognized as a novel animal model for human systemic carnitine deficiency.

Surprisingly, in addition to these pathological features, JVS mice exhibit a remarkably increased number of mitochondria in their heart. However, the biochemical characteristics and physiological functions of these mitochondria cardiac are little known. Here we show that the respiratory activities at state 3 with glutamate plus malate or succinate in the heart mitochondria of JVS mice were greatly decreased to 47% or 77%, respectively, compared with those of control mice. The contents of cytochromes a, a3, b, and c+c1, in the heart mitochondria of these mice were also decreased, to 51%, 45%, and 79%, respectively, of those of the control mice. Oligomycin-sensitive ATPase activity in these mitochondria, however, was increased to about 2 times over that of the control mice. Surprisingly, the ATP-Pi exchange activity of the heart mitochondria of JVS mice was greatly decreased, to 35% of that of control mice. On the other hand, the expression levels of 2 subunits of H+-ATP synthase, i.e., coupling factor 6 and α subunit, in heart mitochondria from control and JVS mice were almost the same. These results indicate that the coordinate regulation of mitochondrial proliferation and gene expression for components of the oxidative phosphorylation system was markedly defective in the heart of JVS mice. Our current results also suggest the presence of a novel regulatory mechanisms of ATP synthase activities in the heart.

Key words  juvenile visceral steatosis (JVS) mice; mitochondria; respiration

MATERIALS AND METHODS

Animals All animals were maintained under specific pathogen-free conditions. Homozygous mutants (jvs/jvs) were produced by mating carnitine-treated homozygous mutant male mice with heterozygous female mice. C57BL/6J mice, the mother strain for the JVS mouse, were used as the control. All animal protocols were carried out according to the Guide for Animal Experimentation, School of Medicine, The University of Tokushima.

Preparation of Mitochondria from Cardiac Tissue by Use of Bacterial Collagengase The mitochondria from cardiac tissue were prepared essentially according to the method of Toth et al., with some modifications. The mouse was first anesthetized with ether, and the cardiac tissue was then excised, immersed in cold MSB medium (225 mM mannitol, 75 mM sucrose, 0.2% (w/v) bovine serum albumin, pH 7.4), and then washed several times with the medium at 0—4 °C. All subsequent operations were performed at 0—4 °C. After having been washed, the tissue was weighed, immediately minced with scissors, and washed again several times with...
MSB medium. The minced and washed tissues were suspended in this medium (10 ml per gram of heart) containing 0.07% (w/v) of collagenase, and incubated for 15 min by stirring 2 to 3 times with a glass rod during the incubation. Homogenization was performed in a glass Potter-Elvehjem homogenizer with 3 up-and-down strokes of a motor-driven pestle set to rotate at 500 rpm. The homogenate was allowed to incubate on ice for 10 min, and then EGTA was added to a final concentration of 1 mM (MSBE medium). The homogenate was centrifuged at 950 x g for 7 min with Beckman JA-20 to sediment cellular debris, and the supernatant was centrifuged at 8700 x g for 11 min. The pelletted mitochondria were gently resuspended in MSBE medium, homogenized with hand with the same way, and centrifuged at 17400 x g for 12 min. Again the pellet was suspended in MSB medium, homogenized by hand with the same way, and centrifuged at 17400 x g for 12 min. The final pellet was rinsed twice with oxygraph medium (250 mM sucrose, 5 mM KH2PO4, 10 mM EGTA, 2 mM HEPES, pH 7.4), suspended in a small amount of this medium, and used as the mitochondrial preparation. We confirmed a purity of the mitochondrial preparation by measuring activities of acid phosphatase (a marker enzyme of lysosome) and glucose-6-phosphatase (a marker enzyme of endoplasmic reticulum). Measurements of oxygen consumption by the isolated mitochondria were carried out within 3 h after preparation.

**Measurement of Oxygen Consumption** The oxygen consumption was measured polarographically at 30°C by using a Clark oxygen electrode (Model 54, Yellow Springs Instruments, Yellow Springs, OH) in air-saturated oxygraph medium in a water-jacketed reaction chamber (1.2 ml). All components of the medium used were essential to obtain a good respiratory index. Respiratory activities were measured with glutamate (10 mM) plus malate (5 mM) or succinate (10 mM) as substrates, and respiration at state 3 and state 4 was measured after the addition of ADP for a final concentration of 250 μM. The addition of 1 μM carbonyl cyanide-m-chlorophenylhydrazone (CCCP) was carried out at state 4. The oxygen concentration in the air-saturated medium at 30°C was assumed to be 445 nmol O2/ml of the medium.6,7

**Measurement of Cytochrome Contents** The contents of cytochromes a + a3, b, and c + c1 in the heart mitochondria were measured with a DU650 spectrophotometer (Beckman Coulter) as the absorbance of reduced minus oxidized mitochondrial suspension at pH 7.4. The amounts of cytochromes were calculated by using the following millimolar extinction coefficients: cytochrome a + a3 (605—630 nm), 16.0; cytochrome b (562—575 nm), 22.0; and cytochrome c + c1 (553—540 nm), 19.1. The contents of cytochromes were expressed as nmol/mg mitochondrial protein.

**Determination of ATPase Activity** Mitochondrial ATPase activity was measured spectrophotometrically in the presence of an ATP-regenerating system essentially as described by Pullman et al. For this assay, the incubation mixture contained the following reagents: 1.88 ml of ATPase reaction medium (25 mM KHCO3, 300 mM sucrose, and 2 mM MgCl2) adjusted to pH 8.2 with 1 M Tris-base, 60 μl of 50 mM phosphoenolpyruvate, 10 μl of 50 mM NADH, 5 μl of pyruvate kinase (1 unit/μl), 5 μl of lactate dehydrogenase (1 unit/μl), and 10 μl of 250 mM ATP (pH 7.4). Production of ADP was determined by monitoring the oxidation of NADH at the wavelength pair of 340—400 nm with a Hitachi two-wavelength spectrophotometer, Model 556. Before addition of the mitochondrial preparation, 10 μl of 0.665 mM ADP (pH 7.4) was added to the incubation mixture to determine the absorbance of NADH oxidation by ADP in the present system. The reaction was started by the addition of 10 μl (2 μg protein) of the mitochondrial preparation, and the reaction was followed for 3 to 5 min. After the incubation, 10 μl of oligomycin solution (1 μg/μl) was added to the incubation mixture to determine oligomycin-sensitive ATPase activity. ATPase activity was expressed as mmol ADP produced/mg protein/min.

**Determination of ATP-Pi Exchange Activity** ATP-Pi exchange activity was measured as described previously. Briefly, the mitochondria (100 μg of protein) were suspended in 0.5 ml of the oxygraph medium described above containing 5 mM MgCl2, 0.08 μg/μl antimycin A, and 2 32P (2 x 10⁶ cpm), and then the reaction was started in the presence or absence of oligomycin (0.015 μg) by adding 8 mM ATP. The reaction was stopped 5 min later by adding 0.2 ml of 40% trichloroacetic acid, and the mixture was then centrifuged at 10000 rpm for 3 min. An aliquot of the supernatant (0.5 ml) was taken for extracting [32P] ATP, and another aliquot was taken for counting total 32P as described previously. ATP-Pi exchange activity was expressed as nmol Pi/mg protein/min.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting** Mitochondria (1—7 μg protein) were treated with Laemmli’s SDS-PAGE sample buffer (62.5 mM Tris—HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol and 5% 2-mercaptoethanol), resolved on a SDS-PAGE gel, and then subjected to Western blotting analysis as described previously. Membranes were probed with anti-CF 6 or anti-α subunit antibodies, and then developed by enhanced chemiluminescence reagents, ECL plus (Amersham Pharmacia Biotech UK Ltd., U.K.), according to the manufacturer’s instructions.

**Other Methods** Anti-α subunit antibody was obtained from Molecular Probes (Eugene, OR). Anti-CF 6 antibody was kindly provided by Dr. K. Magota (Pharmaceutical Research Laboratories, Suntory Institute for Biomedical Research, Osaka, Japan).

Protein concentrations were determined by the method of Lowry et al. The results were analyzed by the unpaired t-test, with the level of significance set at p < 0.05. Reagents used for determination of ATPase activity, ATP-Pi exchange activity, SDS-PAGE, and Western blotting were obtained as described previously.

**RESULTS**

**Mitochondrial Purity** To confirm a purity of the mitochondrial preparations prepared from hearts of control and JVS mice, we examined activities of glucose-6-phosphatase and acid phosphatase, which are marker enzymes of endoplasmic reticulum and lysosome, respectively. The activities of glucose-6-phosphatase in mitochondria prepared from hearts of control and JVS mice by the present method were 0.0046 and 0.0030 μmol/mg protein/min, respectively, which are approximately one % contamination of endoplasmic...
reticulum (0.317 μmol/mg protein/min). Similarly, the activities of acid phosphatase in mitochondria prepared from hearts of control and JVS mice by the present method were 0.0038 and 0.0017 μmol/mg protein/min, respectively, which are approximately one % contamination of lysosome (0.351 μmol/mg protein/min). These data clearly indicate that the present preparation method of mitochondria is excellent and that there is no difference in the purities of mitochondria prepared from control and JVS mice.

Respiratory Activities of the Heart Mitochondria Isolated from Control and JVS Mice To compare the respiratory activities of the heart mitochondria of control and JVS mice, we first developed a method using bacterial collagenase for preparing mitochondria from the heart. As shown in Table 1, the heart mitochondria isolated by the present method possessed a high RCI (respiratory control index) and ADP/O ratios. When glutamate plus malate or succinate was used as the respiratory substrate, RCI and ADP/O ratios of the heart mitochondria of the control mice were 8.1 or 5.1 and 2.7 or 1.5, respectively, which are close to the theoretical values. Heart mitochondria of JVS mice showed values that were almost the same as those of the control mice (Table 1). Thus, these data show that the heart mitochondria isolated from control and JVS mice maintained intact inner and outer mitochondrial membranes. However, respiratory activities at state 3 with glutamate plus malate or with succinate of the heart mitochondria of JVS mice were greatly decreased to 47% or 77%, respectively, compared with those of control mice except the respiration with succinate as substrate (Table 1). In addition, maximal respiratory activities measured after the addition of 1 μM CCCP with glutamate plus malate to the heart mitochondria of JVS mice were also decreased, to 48% of those of control mice.

Contents of Cytochromes in the Heart Mitochondria

Table 1. Respiratory Activities of the Heart Mitochondria Isolated from Control and JVS Mice with Glutamate Plus Malate or Succinate as Substrate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>State 3</th>
<th>State 4</th>
<th>CCCP</th>
<th>ADP/O</th>
<th>RCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate plus malate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>316±39</td>
<td>43±10</td>
<td>190±26</td>
<td>2.7±0.3</td>
<td>8.1±1.9</td>
</tr>
<tr>
<td>JVS</td>
<td>149±48*</td>
<td>17±4*</td>
<td>91±25*</td>
<td>2.8±0.1</td>
<td>9.2±2.5</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>417±47</td>
<td>110±36</td>
<td>312±55</td>
<td>1.5±0.1</td>
<td>5.1±2.1</td>
</tr>
<tr>
<td>JVS</td>
<td>320±22*</td>
<td>124±44</td>
<td>273±59</td>
<td>1.3±0.1*</td>
<td>3.2±1.3**</td>
</tr>
</tbody>
</table>

Respiratory activities of mitochondria were determined as described in Materials and Methods. Each value represents the mean±S.D. for 6 separate experiments with control and JVS mice. *p<0.0001, control mouse versus JVS mouse. **p<0.03, control mouse versus JVS mouse.

Table 2. Contents of Cytochromes in the Heart Mitochondria from Control and JVS Mice

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome (nmol/mg protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a + a\textsubscript{ij}</td>
<td>b</td>
</tr>
<tr>
<td>Control</td>
<td>0.77±0.16 (100%)</td>
<td>0.56±0.07 (100%)</td>
</tr>
<tr>
<td>JVS</td>
<td>0.40±0.05 (51.2%)*</td>
<td>0.25±0.03 (44.8%)*</td>
</tr>
</tbody>
</table>

Each value represents the mean±S.D. for 8 and 6 separate experiments for control and JVS mice, respectively. *p<0.001, control mouse versus JVS mouse. **p<0.01, control mouse versus JVS mouse.

Isolated from Control and JVS Mice As the decreased respiratory activities could possibly have been due to decreases in the contents of respiratory enzyme complexes, we determined the contents of cytochromes in the heart mitochondria of control and JVS mice. As shown in Table 2, the contents of cytochromes a + a\textsubscript{ij}, b, and c + c\textsubscript{i} of the heart mitochondria of JVS mice were also decreased, to 51%, 45%, and 79%, respectively, of those of the control mouse.

Oligomycin-Sensitive ATPase Activity and ATP-Pi Exchange Activity of the Heart Mitochondria Isolated from Control and JVS Mice Next, we investigated the functions of H\textsuperscript+-ATP synthase in the heart mitochondria by measuring oligomycin-sensitive ATPase activity and ATP-Pi exchange activity, which represent the ATP synthesis capacity. In contrast to the decreased respiratory activities and cytochrome contents, oligomycin-sensitive ATPase activity of the mitochondria from JVS mice was increased to about 2 times of that of control mice (Table 3). Surprisingly, however, the ATP-Pi exchange activity was greatly decreased to 35% of that of the control mice (Table 3).

The mammalian H\textsuperscript+-ATP synthase is a supramolecule composed of at least 14 subunits that have a constant stoichiometry. Six of them construct the catalytic site of ATP synthesis called F\textsubscript{1} (subunits α, β, γ, δ, ε and the closely attached ATPase inhibitor protein IF1), and the other 8 forms the energy transduction part called Fo [subunits a, b, c, d, e, CF 6, OSCP (oligomycin sensitivity-conferring protein), and A6L]. Then, we next determined whether the expression level of H\textsuperscript+-ATP synthase was affected in the mitochondria of JVS mouse by measuring the expression levels of 2 of three subunits of H\textsuperscript+-ATP synthase by Western blotting analysis. As shown in Fig. 1, the expression levels of α subunit and CF 6 in the heart mitochondria from control and JVS mice were almost the same.
DISCUSSION

It has been thought that the mitochondrial proliferation in the JVS mouse heart is increased as a result of an energy compensatory mechanism, because carnitine is essential for the transport of long-chain acyl coenzyme A into the mitochondrial matrix, and thereby, carnitine deficiency results in a defect in cellular energy metabolism.\textsuperscript{30,31} However, the facts that the respiratory activities, cytochrome contents, and ATP-Pi exchange activity of the heart mitochondria of JVS mice were greatly decreased as compared with those of control mice indicate that the heart mitochondria of these mice are insufficient to compensate for the decreased energy production in the heart. Recently, Kuwajima \textit{et al.} reported that total content of adenine nucleotides (ATP and ADP) in the JVS mouse heart was about 60% of that in the control mouse heart and that adenylate energy charge in the JVS mouse heart was 63% and 45% of that in the control mouse heart at 4 and 8 weeks after birth, respectively.\textsuperscript{7} The present findings clearly demonstrate that the respiratory and ATP-Pi exchange activities were greatly depressed in the JVS mitochondria compared with those in the control mitochondria. Thus, functional disorders of the oxidative phosphorylation system in the heart mitochondria of the JVS mouse might be one cause of the development of the several pathological features of this mouse, especially of those in the heart.

The regulatory mechanism governing the biogenesis of mitochondrial enzyme complexes in the heart of JVS mice is poorly understood at present. However, it is noteworthy that acetyl-L-carnitine has been shown to be able to stimulate the transcription of mitochondrial DNA such as 12S rRNA and the mRNA of subunit I of cytochrome oxidase under altered metabolic conditions such as in hypothyroidism and senescence rats.\textsuperscript{32,33} Furthermore, it has recently been reported that the long-chain fatty acids suppress the induction of urea-cycle enzyme genes by glucocorticoid action in rat hepatocytes in primary culture.\textsuperscript{34} Therefore, it is conceivable that the long-chain fatty acids not metabolized in the heart are involved in the regulation of the expression of mitochondrial proteins such as cytochromes in the heart of JVS mice. This possibility is now under investigation.

A further point of interest of the present findings is that the oligomycin-sensitive ATPase activity of heart mitochondria of JVS mice was increased to about 2 times over that of the control mouse, whereas the ATP-Pi exchange activity, which represents the ATP synthesis capacity, of the mitochondria of JVS mice was greatly decreased to 35% of that of control mice (Table 3). These findings are extremely interesting because it has been generally thought that the increase or decrease in the oligomycin-sensitive ATPase activity of H\textsuperscript{+}-ATP synthase reflected the increase or decrease in ATP-Pi exchange activity of the enzyme, respectively, and thus, the oligomycin-sensitive ATPase activity of H\textsuperscript{+}-ATP synthase has been taken as a measure of the ATP synthesis capacity of

Table 3. Oligomycin-Sensitive ATPase Activity and ATP-Pi Exchange Activity of the Heart Mitochondria Isolated from Control and JVS Mouse

<table>
<thead>
<tr>
<th></th>
<th>ATPase activity (mmol ADP/mg protein/min)</th>
<th>Oligomycin sensitivity (%)</th>
<th>ATP-Pi exchange activity (nmol Pi/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.37±0.62 (100%)</td>
<td>88.06</td>
<td>59.42±11.72 (100%)</td>
</tr>
<tr>
<td>JVS</td>
<td>8.50±1.11 (195%)*</td>
<td>89.79</td>
<td>20.77±5.72 (35%)**</td>
</tr>
</tbody>
</table>

Oligomycin-sensitive ATPase activities and ATP-Pi exchange activities of the heart mitochondria from control and JVS mouse were measured spectrophotometrically in the presence of an ATP-regenerating system as described in Materials and Methods. The values are means±S.D. for 6 experiments with control and JVS mice. *p<0.001, control mouse versus JVS mouse. **p<0.0001, control mouse versus JVS mouse.
this enzyme. However, the present results clearly show that ATPase activity could not be used as a measure of the ATP synthesis capacity of this enzyme, suggesting the presence of novel regulatory mechanisms of ATP synthase activities in the heart. Furthermore, it should be noted that the contents of 2 subunits of H^-ATP synthase in mitochondria prepared from the heart of JVS mice were almost the same as those of control mice (Fig. 1). Further investigation is required to clarify why in spite of the fact that the content of H^-ATP synthase in the heart mitochondria of JVS mice was similar to that in control mice, ATPase activity in the heart mitochondria of JVS mice was dramatically enhanced, but ATP-Pi exchange activity in the JVS mitochondria was greatly decreased compared with that of the control mice.

Recently, we found that most of the transcripts of the 16 subunits of rat H^-ATP synthase were concertedly and synchronously expressed, having a constant expression pattern irrespective of the tissue or age of the rats. As shown in this present study, the expression levels of α subunit and CF 6 of H^-ATP synthase in the heart mitochondria from control and JVS mice were almost the same (Fig. 1). In addition, we found that the mRNA levels of subunits of H^-ATP synthase complexes such as subunits c(P1), c(P2), β-subunit, and ATPase inhibitor protein IF1 in the heart of JVS mice were also almost the same as those of control mice (K. Morokami et al., unpublished observations.), suggesting that the synchronized transcriptional gene expression of H^-ATP synthase subunits was normally conducted even in the carnitine deficiency, although mitochondrial proliferation, contents of cytochromes, and H^-ATP synthase activities were markedly affected by the carnitine deficiency. Thus, the previous and the present results indicate that JVS mice are useful animal model for studying the regulatory mechanisms of not only mitochondrial biogenesis but also gene expression of subunits for the oxidative phosphorylation system in the mitochondria.

Mitochondria exist in a particular cell type with a characteristic copy number, and mitochondrial number and functions are altered in response to external stimuli in eukaryotes. Although several transcription/replication factors have been shown to regulate mitochondrial gene expression, mechanisms controlling tissue-specific mitochondrial copy number are little known. Recently, peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) was discovered as a regulator of mitochondrial biogenesis in tissues specialized for thermogenesis, such as brown adipose tissue. PGC-1 was shown to stimulate mitochondrial biogenesis and respiration in muscle cells through an induction of uncoupling protein 2 (UCP-2) and through regulation of nuclear respiratory factors (NRFs). PGC-1 was shown to powerfully induce mRNA for NRF-1 and NRF-2α, which are regulators of multiple target genes such as cytochrome c oxidase subunit IV, the b subunit of H^-ATP synthase, as well as that for mitochondrial transcription factor A (mtTFA), a direct regulator of mitochondrial DNA replication/transcription. However, PGC-1 seems unlikely to be a regulator of mitochondrial biogenesis in the heart of JVS mice because mRNA levels of subunits of H^-ATP synthase were almost the same as those of control mice as described above. Thus, these results strongly suggest the presence of some novel heart-specific transcription/replication factors controlling the mitochondrial number, because the increase in mitochondrial number in JVS mice was observed only in the cardiac and skeletal muscle cells. In addition, Bergeron et al. reported that chronic activation of AMP-activated protein kinase (AMPK), which is caused by energetic stress by treatment with the creatine analog β-guanidinopropionic acid (β-GPA), might result in mitochondrial biogenesis in skeletal muscle. They suggested that activation of AMPK also activated NRF-1, which thereby resulted in an increase in the cytochrome c content.

In order to clarify the molecules controlling the mitochondrial number in the heart of JVS mice, we recently performed mRNA differential display analysis with mRNA extracted from the hearts of control and JVS mice, and identified 8 up-regulated genes, one of which was specifically expressed in JVS mice, and one down-regulated gene in JVS mice. We are now investigating the roles of these gene products in controlling the mitochondrial number and in regulating the H^-ATP synthase activities.

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REFERENCES AND NOTES

1) Present address: Department of Microbiology, Kanazawa Medical University, Uchinada, Ishikawa 920–0293, Japan.