Oligomycin sensitivity of mitochondrial F$_{1}$F$_{0}$-ATPase in diabetes-prone BHE/Cdb rats

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Kim, Sook-Bae, and Carolyn D. Berdanier. Oligomycin sensitivity of mitochondrial F$_{1}$F$_{0}$-ATPase in diabetes-prone BHE/Cdb rats. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E702–E707, 1999.—BHE/Cdb and Sprague-Dawley rats differ in their mitochondrial DNA sequence for the ATPase 6 ("subunit a") gene. Base substitutions in this sequence result in the substitution of asparagine for aspartate at position 101 and the substitution of serine for leucine at position 129. Differences in sensitivity to oligomycin were observed. When the isolated F$_{1}$F$_{0}$-ATPase complex was studied and ATPase activity was assessed, that which was isolated from the BHE/Cdb rats was less sensitive to oligomycin inhibition than that which was isolated from the Sprague-Dawley rats. In contrast, when oxygen consumption was measured [oxygen phosphorylation (OXPHOS)] and a dose-response curve was generated with isolated mitochondria from these two strains, there was a shift to the left for the BHE/Cdb rat mitochondria. These mitochondria were more sensitive to oligomycin inhibition of OXPHOS than were mitochondria isolated from Sprague-Dawley rats. The OXPHOS results are consistent with those from human fibroblasts having either a normal or mutated ATPase 6 gene.

oxidative phosphorylation; mitochondria; diabetes

THE BHE/CDB RAT mimics the human with mitochondrial diabetes (1–3). It develops moderate hyperglycemia and impaired glucose tolerance as it ages, and its mitochondrial function deteriorates (1–3, 5, 6). These traits are maternally inherited (20). Detailed studies of these diabetes-prone rats have shown that these rats have less efficient coupling of mitochondrial respiration to ATP synthesis than normal rats (5, 6, 11, 28). Although thyroxine can increase mitochondrial ATP synthesis in normal rats, via an increase in the synthesis and activity of proteins in the various components of oxidative phosphorylation (OXPHOS) (25, 26), it was without effect in BHE/Cdb rats (4). These results suggested that although thyroxine increased mitochondrial protein synthesis, the proteins that were synthesized did not contribute to an increase in ATP production. In fact, with some substrates, the mitochondria appeared uncoupled. This observation could be explained by the presence of a genetic error in the mitochondrially encoded subunits of the F$_{1}$F$_{0}$-ATPase. Recent studies of the BHE/Cdb rat have shown that base substitutions at positions 8204 and 8289 in the mitochondrial ATPase 6 gene exist in this rat (19; C. Herrnstad, unpublished observations). The inferred amino acid substitutions at residues 101 and 129 of F$_{0}$-ATPase "subunit a," which is encoded by the ATPase 6 gene, could have an effect on the functional characteristics of F$_{1}$F$_{0}$-ATPase. Subunit a provides part of the proton channel. The other part is provided by subunit 9 (subunit c), a nuclear-encoded subunit. The inferred amino acid sequences of normal and mutated subunit a suggested that these amino acid substitutions could affect the functional characteristics of the F$_{1}$F$_{0}$-ATPase.

This would be consistent with early reports of mutated Escherichia coli F$_{1}$F$_{0}$-ATPase (7, 13, 23). We have already reported that hepatic mitochondria from BHE/Cdb rats were more responsive to the suppression of OXPHOS by the calcium ion than mitochondria from Sprague-Dawley rats (17). In the present work, we report on the strain differences in oligomycin sensitivity of F$_{1}$F$_{0}$-ATPase and of the isolated mitochondria. We hypothesized that the amino acid substitutions in the subunit a would increase the sensitivity of mitochondria to oligomycin. Oligomycin blocks proton conductance primarily through its binding to the oligomycin-conferring protein found in the stalk of the F$_{1}$F$_{0}$-ATPase complex. We hypothesized that oligomycin binding would also involve the F$_{0}$ portion of ATPase. Hence, we examined both the activity of F$_{1}$F$_{0}$-ATPase complex and OXPHOS with respect to the response to oligomycin. Whereas the isolated F$_{1}$F$_{0}$-ATPase from the BHE/Cdb rats was less sensitive to oligomycin inhibition than that from the Sprague-Dawley rats, when isolated mitochondria were studied, the reverse was found.

MATERIALS AND METHODS

Animals and diets. Two strains of rats were used: BHE/Cdb (UGA colony) and Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). In each study, the rats were fed a standard stock diet (laboratory animal chow, Ralston Purina, St. Louis, MN) and killed at 150 days of age. They were cared for in accordance with the guidelines of the National Institutes of Health Publication 88–23 Guide for the Care and Use of Laboratory Animals.

Preparation of liver mitochondria. Freshly isolated liver mitochondria from BHE/Cdb and Sprague-Dawley rats were separated from the mitoplasts by isolation in isolation medium (H-medium that contained 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, and 0.5 mg/ml defatted BSA) were treated with 4 mg/ml digitonin on ice for 20 min with stirring. This mixture was then diluted 3:1 with H-medium and centrifuged at 10,000 g (10 min, 0–4°C). The resulting sediment was resuspended in one-half of the original volume in H-medium and centrifuged as described previously. These mitoplasts were resuspended in H-medium at 50 mg/ml, diluted 1:25 (vol/vol) in cold deionized water to 2 mg/ml, and centrifuged at 10,000 g for 15 min. The sediment was resuspended to 50 mg/ml in cold, deionized water and
sonicated with the large probe at 95% maximal intensity for 2 min total time in 15-s intervals at 0–4°C. Large mitochondrial fragments were removed by centrifugation at 10,000 g for 30 min at 0–4°C. The sediment was resuspended to a final concentration of 50 mg/ml with H-medium. SMP were added at a concentration of 5 mg of protein/ml with gentle stirring to PEG buffer [20 mM potassium phosphate, 5 mM EDTA, and 20% (vol/vol) glycerol (pH 7.4)] at 0°C. Then 1% n-heptyl β-thioglucoside was added, and the mixture was stirred gently for 5 min. The suspension was diluted with an equal volume of PEG buffer and centrifuged at 105,000 g for 30 min. The resulting supernatant was concentrated in a Centricon 10 (Centricon Microconcentrator, Amicon), and then the solvent was changed by adding an equal volume of PEG buffer and centrifuged to its original volume in the Centricon cell. The procedure was repeated two times. All procedures were carried out at 0°C. The solution (10 mg of protein) was applied to MonoQ-HR 10/10 column (Pharmacia), anion-exchange HPLC, which was equilibrated with PEG buffer. Elution was carried out with a linear gradient of 0–100% 250 mM potassium phosphate, 5 mM EDTA, 1 M KCl, and 20% glycerol at pH 7.4 for 60 min. Fractions containing F1F0-ATPase were collected and centrifuged at 155,000 g for 2 h at 20°C, and the precipitate obtained was suspended in a minimum volume of PEG buffer.

Protein determination. Mitochondria and mitoplast protein were measured by the biuret method. SMP and purified F1F0-ATPase protein were measured by the Bradford method. BSA was used as standard in both cases.

Determination of ATPase activity. ATPase was assayed by a modification of the method of Pullman and Monroy (24). The basic incubation mixture (1 ml) consisted of 50 mM Tris·HCl (pH 8.0), 3.3 mM MgCl2, 2 mg antimycin A, 1 mM ATP, and 0.3 mM NADH, 1 mM phosphoeno pyruvate, 5 U of lactate dehydrogenase, and 2.5 U of pyruvate kinase. The reaction was initiated by the addition of 10–50 µl of the sample to be measured. Oxidation of NADH was followed spectrophotometrically at 340 nm at a constant temperature of 30°C. The sensitivity of the ATPase to oligomycin was measured by the addition of 10 µl of 650 µg/ml oligomycin solution. Enzyme activity was expressed in terms of micromoles of ATP hydrolyzed per minute per milligram of protein, which is equal to the micromoles of NADH oxidized per minute per milligram of protein. Oligomycin sensitivity was expressed as the percent reduction in ATPase activity with the addition of oligomycin. To determine the ability of a monoclonal antibody to inhibit the enzyme activity, 100 µg of monoclonal antibody in PBS were incubated with 10 µg purified F1F0 ATPase before the assay of the ATPase activity.

Determination of OXPHOS. Oxygen consumption was determined with an oxygen electrode (model 5331, Yellow Springs Instrument, Yellow Springs, OH) with a 2.5-ml chamber and oxygen meter (UGA Instrument Design Group, Athens, GA). The reaction chamber was fitted with a magnetic stirrer, and temperature was controlled at 25°C. The respiration medium consisted of 75 mM glycine, 10 mM phosphate buffer (pH 7.4), 75 mM KCl, and 10 mM Tris·HCl (pH 7.2). The medium was equilibrated with air at 25°C. After the medium was placed in the incubation chamber, subsequent additions were made with Hamilton syringes through the capillary aperture on the top of the apparatus. The entry port was kept sealed at all other times. The following reagents were stored frozen until needed: 25 mM ADP (pH 6.8), 0.65 M succinate (pH 7.2), and oligomycin (650 µg/ml). In a typical run, freshly isolated mitochondria (2.5 mg protein) were added to the incubation medium containing 5 mM succinate. After ~2 min, 375 nmol of ADP were added to stimulate respiration. This was repeated at least twice. Then, graded amounts (0.02–0.1 µg/ml mitochondrial protein) of oligomycin were added with the ADP, and respiration was again determined. A separate run for each level of oligomycin was performed. State 3 and state 4 oxygen consumption rates were calculated according to Chance and Williams (10). Respiratory control ratios and ADP-to-O ratios were calculated according to Estabrook (12).

Statistics. Where appropriate, the means were compared with Student’s t-test. P < 0.05 was considered significant.

RESULTS

The assessment of the strain differences in ATPase activity at each step of the F1F0-ATPase isolation and purification is shown in Table 1. HPLC and electrophoresis were used for this purification. The elution patterns for the ATPase from the two strains are shown in Fig. 1. The strains differed in this pattern probably due to small differences in the amino acids that comprise the various subunits of the complex. Fraction 36 contained the ATPase in the Sprague-Dawley rats, whereas fraction 33 contained the enzyme complex in the BHE/Cdb rats. Between fractions 10 and 20, there were additional peaks in the extracts. The identity of these peaks is unknown. With respect to the data presented in Table 1, it should be noted that only one level of oligomycin was used to assess its effect on ATPase activity. The level used was 65 µg/ml reaction volume. This level of oligomycin was the highest level of inhibition used in the studies of OXPHOS by the isolated mitochondria. As the ATPase was purified, its specific activity decreased by 10.220.3% on June 10, 2017 http://ajpendo.physiology.org/ Downloaded from

Table 1. Summary of purification of hepatic mitochondria F1F0-ATPase in BHE/ Cdb and SD rats

<table>
<thead>
<tr>
<th></th>
<th>ATPase Sp. Act., µmol·min⁻¹·mg protein⁻¹</th>
<th>Oligomycin Sensitivity, %</th>
<th>Purification, x-fold</th>
<th>Total Protein, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BHE/Cdb</td>
<td>SD</td>
<td>BHE/Cdb</td>
<td>SD</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.35</td>
<td>0.36</td>
<td>35</td>
<td>95</td>
</tr>
<tr>
<td>Mitoplasts</td>
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<td>0.36</td>
<td>35</td>
<td>95</td>
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<tr>
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<td>11.40</td>
<td>35</td>
<td>98</td>
</tr>
</tbody>
</table>

ATPase activity was measured before and after addition of 10 µl of oligomycin (650 µg/ml): % loss in activity due to oligomycin addition. SD, Sprague-Dawley rats; SMP, submitochondrial particles; HTG, n-heptyl β-thioglucoside; Sp. act., specific activity.
activity increased as expected. By the completion of the treatment of the extract with the Centricon, there was an ~32-fold increase in ATPase activity in the extract. There was a corresponding decrease in the amount of protein, and this was expected as well. Regardless of the level of purification, there was a strain difference in sensitivity of the enzyme to oligomycin inhibition. The ATPase from the BHE/Cdb rats was less sensitive to oligomycin inhibition than was the ATPase from the Sprague-Dawley rats. This strain difference was observed at each step of the isolation. Electrophoresis of the final extract showed that the subunits of the ATPase separated in stoichiometric amounts. The bands that were separated from the two strains were similar. With the use of antibodies produced to peptides synthesized to replicate the hydrophilic portion of the ATPase subunit a, a progressive inhibition of the ATPase was shown for both strains (Fig. 2). The maximal inhibition of ATPase activity by the addition of these antibodies was 20% with 60 min of incubation.

Figs. 3, 4, 5, and 6 show the effects of graded additions of oligomycin to mitochondria being assessed
for their OXPHOS performance. In contrast to the results of the study of the effect of oligomycin on ATPase activity, there was a shift to the left in the response of isolated mitochondria from the BHE/Cdb rats. At doses from 0 to 0.04 µg/mg protein, BHE/Cdb mitochondria were more responsive to the inhibitory action of oligomycin with respect to state 3 respiration than were mitochondria from Sprague-Dawley rats. At the highest level, this strain difference reversed itself, although the difference between the strains was not statistically significant.

DISCUSSION

This work is of interest because it shows a strain difference in response to oligomycin that was dependent on the assay used as well as the dose of oligomycin used. When the ATPase assay was used, the mitochondria and the purified enzyme from the BHE/Cdb rats appeared to be less sensitive than that from Sprague-Dawley rats. When OXPHOS was assessed, the reverse was observed. That is, the BHE/Cdb mitochondria appeared to be more sensitive to oligomycin inhibition than mitochondria from Sprague-Dawley rats. On closer examination of these two data sets, however, it is apparent that this contrast in results might be due to the dose of drug used rather than any difference in response to oligomycin by the isolated ATPase and the OXPHOS measurements. At the highest dose of oligomycin, OXPHOS was inhibited in mitochondria from both strains. Because OXPHOS assessment measures more than just the activity of the F1F0-ATPase, the lack of a significant strain difference at the highest oligomycin dose (the dose used when ATPase activity was determined) could be understood. In addition, the mitochon-
drial preparations might have been contaminated with other ATPase-containing organelles. These would contribute ADP to the media and contribute an error term to the OXPHOS measurement. However, electron microscopy of the mitochondrial preparations showed that such contamination was very small (<1%). Mitochondrial preparations from both strains were similarly affected. At the lower levels of oligomycin (0–0.04 µg/mg mitochondrial protein), the strain differences in state 3 oxygen consumption were observed, and this shift to the left indicates that BHE/Cdb mitochondria were respiring far slower than Sprague-Dawley mitochondria and that this rate of respiration was further slowed by increasing levels of oligomycin. Thus, regardless of the measurements made, there definitely was a difference due to strain in the responsiveness to oligomycin.

How does this relate to the known genetic defect in the BHE/Cdb mitochondrial ATPase 6 gene? Our observation of a left shift in oligomycin sensitivity is consistent with the report of Vazquez-Memiji et al. (27). These workers studied fibroblasts from humans with either a T8993G or a T8993C point mutation in the ATPase 6 gene. This mutation is within 30 bp of the mutation we previously reported on their increased sensitivity to calcium ion (17) and on strain differences in OXPHOS in animals fed different carbohydrates and fats (11, 16, 28). Saturated fat in the diet potentiates the strain difference in OXPHOS (16). Others (8) have also reported that increases in cholesterol content can affect mitochondrial ATPase activity.

Altogether, the present data on differences in oligomycin sensitivity plus those previously reported provide considerable insight into the role mitochondria play in the control of metabolism, especially the metabolism of glucose.

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