The Effects of Iron Deficiency on the Respiratory Function and Cytochrome Content of Rat Heart Mitochondria

LYNDA BLAYNEY, PH.D., ROYSTON BAILEY-WOOD, PH.D., ALAN JACOBS, M.D., ANDREW HENDERSON, M.B., AND JOHN MUIR, D.M.

SUMMARY We have examined the effects of total body iron deficiency on the function of mitochondria isolated from rat hearts. Male Wistar rats were weaned at 21 days and divided into an experimental iron-deficient group and a control group. Both groups received identical diet but an iron supplement (180 mg of ferrous sulfate per kg of diet) was added for the control group. Rats were studied at 7 and 14 weeks. Iron-deficient rats weighed less than controls but showed significantly increased ventricle to body weight ratio at both 7 and 14 weeks, indicating relative cardiac hypertrophy. Isolated mitochondrial fractions from iron-deficient and control rats contained similar proportions of whole homogenate protein and succinic cytochrome c reductase activity, indicating that the fractions isolated from the experimental and control rats were comparable. In iron-deficient rats NADH cytochrome c reductase, succinic cytochrome c reductase, succinic dehydrogenase, and NADH ferricyanide oxidoreductase activities were all significantly reduced at 7 and 14 weeks. Cytochrome c oxidase activity was significantly reduced only at 14 weeks as were the concentrations of cytochromes a, c1, and b. The rate of oxygen uptake by mitochondria was significantly lower at both 7 and 14 weeks but the P/O ratio was unaltered. We conclude that iron deficiency is associated with impairment of myocardial mitochondrial electron transport.

ANEMIA may contribute clinically to the development of angina and of congestive heart failure, and it has been shown that cardiac enlargement correlates reversibly with the presence and severity of anemia. These effects may be attributed to the reduced capacity of the blood to provide oxygen for myocardial metabolism, and to the increased volume overload imposed on the heart with consequent dilation and hypertrophy. In addition, the presence of anemia or, more specifically, of iron deficiency, may itself lead to intrinsic changes in myocardial metabolic function.

Other workers have reported cardiac hypertrophy (increased heart to body weight ratio) in iron-deficient rats. The increase in heart weight is due to muscle, and the associated increase in mitochondrial mass may be sufficient to account for 75% of the total increase in heart weight. Hypertrophy regressed with iron therapy and the increased ratio of mitochondrial to myofibrillar area seen on electron microscopic section returned to normal. Despite dramatic changes in mitochondrial morphology the P/O ratios remained normal, indicating that there was no loss of sites of oxidative phosphorylation, although iron deficiency does impair oxidative phosphorylation at site I in the yeast Torulopsis utilis. Site I depends on the presence of non-heme iron. The cytochrome components of the mitochondrial respiratory chain depend also on iron complexed as heme. Iron is thus an integral part of the mitochondrial respiratory system. A reduction of cytochrome c content previously has been demonstrated in whole samples of heart and other organs, and succinic dehydrogenase activity has been shown to be depressed in whole heart tissue slices from iron-deficient rats. Mitochondrial preparations have been studied from the intestinal mucosa in iron deficiency and showed a reduction of succinic dehydrogenase and cytochrome c oxidase activities, but enzyme activities and cytochrome contents have not been examined previously in mitochondria isolated from the heart in iron deficiency. The present study was designed to measure these, using rats deprived of dietary iron for different periods of time.

Methods

Male Wistar rats were kept in plastic cages and given deionized water to drink. Iron-deficient and iron-supplemented diets were given ad libitum from weaning at 21 days. The diet was that described by McCall et al. The control rats received the same diet supplemented with 180 mg of iron (FeSO₄·7H₂O) per kg. Measurements relating to the iron status of the rats used in the experiments are given in Table 1.

PREPARATION OF SUBCELLULAR FRACTIONS

Before the animals were killed they were allowed water but deprived of food for 20 hours. They were killed by a blow to the neck. The hearts were placed immediately into ice-cold isolation buffer which contained 0.18 m KCl, 10 mm ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin, pH 7.0, adjusted with tromethamine (Tris). The wet weight of the ventricles was recorded after removal of fat, connective tissue, and the atria. Ventricles were chopped into 1-mm cubes with chilled scissors in an ice-cold beaker. The ventricles from two hearts were pooled for each experiment; 10 ml of isolation buffer were added per g of tissue, and the suspension was homogenized with a Tri-R Stir R homogenizer (setting 6) for four strokes in a glass beaker.
homogenizer with a Teflon pestle. Part of the resulting homogenate was kept, diluted with isolation buffer, and assayed as the whole homogenate. Mitochondria were prepared from the remainder by the method of Lindenmayer et al.\textsuperscript{14} The final mitochondrial pellet was resuspended in 4 ml of isolation buffer and used immediately. Whole mitochondria were used for the assays. Preliminary work in this laboratory has shown that methods of disruption often used to enhance enzyme activity (such as freezing and thawing, treatment with deoxycholate, or sonication) do not alter the activity of each enzyme to the same extent. The use of such methods in the present study might therefore have introduced artifacts due to differences in the response to disruption of mitochondria isolated from control and from iron-deficient rats.

**ENZYME ASSAYS**

All mitochondrial enzyme activities were assayed with a Unicam SP 1800 recording spectrophotometer.

Succinic dehydrogenase ([EC1.3.99.1] (succinate:dichlorophenyl indophenol oxidoreductase)] was assayed by the method of Redfearn and Dixon.\textsuperscript{13} NADH cytochrome c reductase [([EC 1.6.2.1) (NADH + H+:cytochrome c oxidoreductase)], and succinic cytochrome c reductase were measured by the method of Mahler;\textsuperscript{14} NADH ferricyanide oxidoreductase by the method of King and Howard;\textsuperscript{15} and cytochrome c oxidase [([EC 1.9.3.1) (ferrocytochrome c oxygen oxidoreductase)] by following the oxidation of reduced cytochrome c (final concentration = 2.155 mM in 100 mM Tris-acetate buffer, pH 6.0) at 550 nm.

Specific activities were expressed as units of enzyme per mg of protein, where 1 enzyme unit was taken as that catalyzing the transformation of 1 \( \mu \)mol of substrate per min. The activity of cytochrome c oxidase was expressed as the first-order rate constant \( K \text{sec}^{-1} \) per mg of protein.

**CYTOCHROME SPECTRA**

Cytochrome spectra were recorded in a Unicam SP 1800 recording spectrophotometer using cuvettes with a light path of 1 cm. The cytochrome content of the mitochondrial fraction was estimated from difference spectra (air-oxidized minus dithionite-reduced). Air-oxidized preparations were obtained by vigorously shaking 1 ml of mitochondrial fraction with air. Dithionite-reduced preparations were obtained by the addition of a few crystals of dithionite to a cuvette. The cytochrome \( a_2 \) concentration was estimated from the change in absorbance between the trough at 630 nm and the peak at 605 nm, using the molar extinction coefficient \( E = 12.0 \times 10^3 \). Cytochrome \( b \) and \( c \) concentrations were calculated from the change in absorbance between the respective peaks at 563 nm and 554 nm, the molar extinction coefficients being 14.3 \( \times 10^3 \) and 18.8 \( \times 10^3 \), respectively.\textsuperscript{16} The final concentrations were expressed as nmol/mg mitochondrial protein.

**OXIDATIVE PHOSPHORYLATION AND OXYGEN UPTAKE**

Oxygen uptake was measured polarographically with a Gilson Oxygraph fitted with a Clark oxygen electrode. The reaction mixture (final volume = 3 ml) contained 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 8.5 mM \( K_2HPO_4 \), and the mitochondrial fraction to give a final protein concentration of approximately 1 mg/ml.13 Glutamate, 10 \( \mu \)l (final concentration = 50 mM), was added after the endogenous rate of respiration had been recorded for a few minutes, and after a further 3 minutes 10 \( \mu \)l of 50 mM ADP were added. The P/O ratio was calculated from the ADP/O ratio. Oxygen uptake was defined as the state 3 respiration rate.

**PROTEIN**

Protein concentrations were measured by the method of Lowry et al.\textsuperscript{,17} using bovine serum albumin, Cohn fraction V (Sigma), as the standard.

**CHARACTERIZATION OF MITOCHONDRIAL FRACTION**

The mitochondrial fractions were characterized to ensure that they were comparable in control and iron-deficient rats (Table 2). The proportion of the initial whole homogenate protein which was isolated in the mitochondrial fraction was

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**Table 1** Measurements Relating to Iron status of the Control and Iron-Deficient Rats Used in Experiments

<table>
<thead>
<tr>
<th></th>
<th>Normal (( n = 12 ))</th>
<th>Iron-deficient (( n = 12 ))</th>
<th>Normal (( n = 6 ))</th>
<th>Iron-deficient (( n = 8 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>15.5 ± 0.80</td>
<td>8.1 ± 0.90</td>
<td>14.1 ± 0.29</td>
<td>6.6 ± 0.90</td>
</tr>
<tr>
<td>Serum iron (( \mu )g/100 ml)</td>
<td>173 ± 11.0</td>
<td>52 ± 10.7</td>
<td>150 ± 10.0</td>
<td>41 ± 7.7</td>
</tr>
<tr>
<td>Total iron-binding capacity (( \mu )g/100 ml)</td>
<td>453 ± 31</td>
<td>711 ± 32</td>
<td>460 ± 49</td>
<td>790 ± 33</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>38.0 ± 1.4</td>
<td>7.2 ± 0.3</td>
<td>32.0 ± 1.5</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Liver non-iron (( \mu )g/100 g)</td>
<td>8.75 ± 0.73</td>
<td>1.3 ± 0.42</td>
<td>10.4 ± 0.35</td>
<td>1.12 ± 0.55</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM; \( n \) = number of rats.

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found to be the same at each time in the two experimental groups. Similarly the mitochondrial marker enzyme activity in the mitochondrial fraction was found to be the same proportion of that measured for the initial whole homogenate in both groups. Thus the mitochondrial fractions appeared to be similar for the control and iron-deficient groups, with respect to the proportion of whole homogenate protein and marker enzyme activity recovered.

BIOCHEMICALS
NADH (Sigma grade), horse heart cytochrome c (type III), and ADP (disodium salt from equine muscle) were obtained from Sigma.

STATISTICAL ANALYSIS
The results were analyzed with Student’s t-test. All results are presented as the mean ± SEM of n experiments.

### TABLE 3 Body Weight, Wet Weight of Ventricles, and Body Weight to Ventricle Weight Ratio of 7- and 14-Week Control and Iron-Deficient Rats

<table>
<thead>
<tr>
<th></th>
<th>7 weeks</th>
<th></th>
<th>14 weeks</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Iron-deficient</td>
<td>Control</td>
<td>Iron-deficient</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>184.64</td>
<td>±7.8</td>
<td>110</td>
<td>±7.8</td>
</tr>
<tr>
<td>Ventricle wt (g)</td>
<td>0.65</td>
<td>±0.04</td>
<td>0.51</td>
<td>±0.05</td>
</tr>
<tr>
<td>Ventricle/body wt (ratio, %)</td>
<td>0.351</td>
<td>±0.0165</td>
<td>0.450</td>
<td>±0.0360</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM; there were six rats in each group. NS = not significant.
and 14 weeks. Cytochrome c oxidase activity was significantly reduced only at 14 weeks.

MITOCHONDRIAL CYTOCHROME CONTENT

Cytochrome a<sub>2</sub>, b, and c<sub>1</sub> were all significantly reduced in concentration at 14 weeks but not at 7 weeks (Table 5).

OXIDATIVE PHOSPHORYLATION AND OXYGEN UPTAKE

The rate of oxygen uptake was significantly reduced in iron-deficient rats at both 7 and 14 weeks (Table 6). The P/O ratio was slightly reduced at 14 weeks, but the difference did not reach statistical significance.

Discussion

We have demonstrated impairment of respiratory enzyme activities but not of oxidative phosphorylation in mitochondrial preparations from hearts of rats with iron deficiency anemia associated with relative cardiac hypertrophy. The decline in enzyme activities and in cytochrome concentrations was more marked at 14 than at 7 weeks, indicating a progressive deterioration. The decreases in cytochrome content could be due to limitation of heme biosynthesis from lack of iron. The decrease in respiratory enzyme activity may be related to a number of factors.

The decline in activity of the respiratory enzymes (except cytochrome c oxidase) and of overall oxygen uptake preceded the decline in content of cytochromes a<sub>2</sub>, b, and c<sub>1</sub>. The decline in activity therefore cannot readily be ascribed to a reduction in concentration of any one of the cytochromes to rate-limiting levels. However, each of these enzyme systems (except cytochrome c oxidase) contain non-heme iron in addition to cytochromes and it was not possible to obtain measurements of non-heme iron. It remains possible that respiratory enzyme activity was depressed by critical shortage of non-heme iron. Cytochrome c oxidase activity is not dependent on non-heme iron and notably was decreased only at 14 weeks in association with a reduction in cytochrome a<sub>2</sub> concentration at this time.

Structural organization and integrity of the inner mitochondrial membrane is important to function. Goodman et al. observed considerable morphological change in cardiac mitochondria in iron deficiency. These changes might alter membrane permeability to electron donors and acceptors used to assay the respiratory enzyme activity. However, membrane disarray, by increasing permeability, would seem more likely to enhance than to impair the measured activity. It is possible too that the reduction in specific activity might be due in part to a redistribution of mitochondrial protein at the expense of the inner membrane where the respiratory activity resides. Artifacts due to differences in preparation of the mitochondrial fractions would appear to have been excluded (see Methods).

### TABLE 4 Activity of the Respiratory Enzymes in the Mitochondrial Fraction

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>7 weeks</th>
<th>14 weeks</th>
<th>7 weeks</th>
<th>14 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Iron-deficient</td>
<td>Control</td>
<td>Iron-deficient</td>
</tr>
<tr>
<td>Succinic cytochrome c reductase</td>
<td>233 ±7.4</td>
<td>143 ±15.5</td>
<td>302 ±10.3</td>
<td>133 ±18.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.005</td>
<td></td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>NADH cytochrome c reductase</td>
<td>283 ±35.9</td>
<td>179 ±10.2</td>
<td>368 ±34.8</td>
<td>170 ±33.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.05</td>
<td></td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>NADH ferricyanide oxidoreductase</td>
<td>6223 ±424</td>
<td>4006 ±222</td>
<td>6290 ±333</td>
<td>4398 ±469</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td></td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>190 ±22.3</td>
<td>120 ±16.3</td>
<td>230 ±35.7</td>
<td>100 ±24.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.05</td>
<td></td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>1.67 ±0.12</td>
<td>1.70 ±0.07</td>
<td>0.88 ±0.04</td>
<td>0.51 ±0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td></td>
<td>P &lt; 0.025</td>
</tr>
</tbody>
</table>

The assay procedures and preparation of the mitochondrial fraction are given in Methods. Enzyme activities are expressed as nmol of substrate per mg of protein per minute, but cytochrome c oxidase is expressed as the first-order rate constant k sec<sup>-1</sup> per mg of protein. There were six rats in each group. NS = not significant.

### TABLE 5 Concentration of Cytochromes a<sub>2</sub>, b, and c<sub>1</sub> in the Mitochondrial Fraction

<table>
<thead>
<tr>
<th>Cytochrome</th>
<th>7 weeks</th>
<th>14 weeks</th>
<th>7 weeks</th>
<th>14 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Iron-deficient</td>
<td>Control</td>
<td>Iron-deficient</td>
</tr>
<tr>
<td>a&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.50 ±0.037</td>
<td>0.41 ±0.045</td>
<td>0.72 ±0.017</td>
<td>0.52 ±0.06</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>P &lt; 0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>0.57 ±0.041</td>
<td>0.41 ±0.045</td>
<td>0.78 ±0.058</td>
<td>0.55 ±0.069</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0.37 ±0.030</td>
<td>0.27 ±0.030</td>
<td>0.58 ±0.075</td>
<td>0.33 ±0.02</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>P &lt; 0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS = not significant; there were six rats in each group. The calculation of concentration and the preparation of the mitochondrial fraction are given in Methods.
The etiology of the changes in mitochondrial function observed in the present study is likely to be complex. Not only is iron an integral part of the mitochondrial respiratory system but there seems little doubt that iron deficiency anemia causes cardiac hypertrophy. It could be argued that the relative hypertrophy observed reflected generalized body wasting, but other organ to body weight ratios (e.g., liver to body weight) remained constant and no increase in heart to body weight ratio was seen in diet-restricted, weight-matched controls. There thus appears to be true cardiac hypertrophy. A contributory factor in the development of cardiac hypertrophy might have been the volume overload resulting from iron deficiency anemia. In mechanically induced cardiac hypertrophy it is well documented that the mitochondria enlarge, but studies of mitochondrial oxygen uptake, and P/O ratios have given conflicting results, due possibly to differences in the type of experimental model used, the stage of hypertrophy examined, and methods of preparing the mitochondrial fractions. In iron deficiency the further possibility arises that iron itself could contribute to cardiac hypertrophy if the stimulus to hypertrophy is indeed related to a change in the balance of supply of ATP to demand as has been suggested.

References

27. Meerson FZ, Pomoritskaya VS: Role of high energy phosphate compounds in the development of cardiac hypertrophy. J Mol Cell Cardiol 4: 571-597, 1972

Erratum

The correct name of the author of the article honoring Dr. Maurice B. Visscher [Circ. Res. 39: 295-296 (September), 1976] is I.J. Fox. Dr. Fox is a professor in the Department of Physiology at the University of Minnesota Medical School in Minneapolis.
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