Relationship between Structure and Fatty Acid Metabolism in Mitochondria Isolated from Ischemic Rat Hearts

DANIELLE FEUVRAY AND JEAN PLOUËT

SUMMARY We studied mitochondrial structure and intermediates of fatty acid metabolism in mitochondria isolated from ischemic hearts. By electron microscopy, no structural difference was detected between mitochondria isolated from control hearts and from ischemic hearts receiving glucose as the only substrate. However, major differences were observed in mitochondria obtained from control and ischemic hearts receiving both glucose and palmitate. These hearts contained a higher portion of damaged mitochondria. However, measurements of marker enzyme activities failed to show that more mitochondria were lost during the isolation procedure in ischemic than in control hearts. Many densely staining areas (or amorphous densities) were observed in the isolated mitochondria of ischemic hearts. These amorphous densities have an appearance similar to that observed in the intact ischemic heart under the same perfusion conditions. Levels of long-chain acyl-CoA in mitochondria isolated from hearts receiving glucose alone were practically the same for control and ischemic hearts and were only slightly increased in mitochondria of ischemic hearts receiving both glucose and palmitate. On the other hand, levels of long-chain acyl carnitine in mitochondria of ischemic hearts were twice those found in control hearts. The mitochondrial level of long-chain acyl carnitine was approximately four times higher in the ischemic hearts receiving palmitate compared to those receiving no palmitate. This rise in long-chain acyl carnitine levels in mitochondria isolated from ischemic hearts receiving palmitate may be related to modifications of the mitochondrial structure and to the appearance of amorphous densities.


STRIKING changes in mitochondrial morphology, including formation of intramitochondrial densities, occur in severe myocardial ischemic injury (Jennings et al., 1965; Jennings and Ganote, 1972; de Leiris and Feuvray, 1977; Schaper et al., 1979). Mitochondrial densities have been divided into two basic categories: granular and amorphous types. The granular densities, which develop on reflow following ischemia, are composed of calcium phosphate (Jennings and Ganote, 1972). On the other hand, the nature of the amorphous densities, which represent a consistent feature of irreversible injury (Jennings and Ganote, 1976; Schaper et al., 1979), is not clear. Jennings and Ganote (1976) suggested that these osmiophilic structures contained lipid, and recent studies suggest that they consist of aggregates of organic material containing both lipid and denatured protein (Hagler et al., 1979). However, no calcium or phosphorus could be detected in the amorphous matrix densities.

Thus, the biochemical composition of the amorphous densities developed in isolated ischemic rat hearts only when fatty acids were provided in the perfusate. Their appearance correlated with chemically determined high levels of long-chain acyl derivatives of CoA and carnitine (Feuvray, in press), and was associated with a rapid deterioration of mechanical function. Although tissue levels of both long-chain acyl-CoA and acyl carnitine increase in ischemic rat hearts, only the increase in acyl carnitine is proportional to the amount of exogenous fatty acid available (Whitmer et al., 1978). This increase in acyl carnitine is associated largely with the mitochondria in ischemic hearts (Idell-Wenger et al., 1978).

The purpose of the present study was to determine the association between morphological changes and levels of acyl derivatives of CoA and carnitine in mitochondria isolated from aerobic and ischemic rat hearts perfused with or without a solution containing a high level of fatty acid. The ultrastructure of the isolated mitochondria was studied systematically in the different experimental conditions.

Methods

Heart Perfusion

Male rats of the Wistar strain weighing 280–320 g were anesthetized with pentothal (5 mg/100 g body weight, ip). Hearts were excised, the aortas cannulated and perfused for a 10-minute washout period by the Langendorff technique.
The hearts then were perfused either as working hearts (Neely et al., 1967) for 65 minutes (control) or for 5 minutes as working hearts followed by 60 minutes of ischemia (ischemic). Whole-heart ischemia was induced by use of a one-way valve in the aortic outflow (Neely et al., 1973) in which coronary flow initially was reduced by 60%. The perfusate was Krebs bicarbonate buffer gassed with a 95% O2-5% CO2 mixture. The buffer contained either 11 mM glucose plus 3% bovine serum albumin (Sigma, essentially fatty-acid free, fraction V) or 11 mM glucose and 1.5 mM palmitate bound to 3% bovine serum albumin. Palmitate was bound to albumin, as described earlier (de Leiris and Feuvray, 1977). The perfusate (200 ml) was recirculated. Ventricular function was estimated by the product of peak systolic pressure and heart rate as previously described (Feuvray et al., 1979). Following the initial reduction of coronary flow, the rate of decline in pressure development was faster in hearts receiving palmitate than in those receiving no palmitate. Since coronary flow depends on peak systolic pressure in this ischemic preparation, the decrease in pressure reduced coronary flow further. Thus, deterioration of function was more severe in ischemic hearts receiving palmitate.

**Tissue Preparation, Isolation of Mitochondria and Assays**

Some hearts were removed from the animals and processed immediately (nonperfused hearts) and others were perfused under aerobic control or ischemic conditions. The hearts were minced and homogenized in ice-cold MSE buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, 5 mM 4-morpholinopropanesulfonic acid, pH 7.4) with a Polytron tissue homogenizer for two 10-second periods (Idell-Wenger et al., 1978). A sample of the homogenate was processed immediately with the homogenizer for two 20-second periods with the Polytron rheostat set at full speed. One aliquot of the homogenate was stored in liquid nitrogen for determination of CoA and carnitine and another aliquot was kept at −20°C for the determination of total protein and marker enzyme activities.

The remaining homogenate was centrifuged at 500 g for 10 minutes, the pellet removed, and the supernatant recentrifuged for another 10 minutes at the same speed. The resultant supernatant was centrifuged at 8000 g for 10 minutes to obtain the mitochondrial pellet, which was washed once before final resuspension in a small volume of MSE buffer.

An aliquot of the mitochondrial suspension was fixed immediately for electron microscopy. The remaining mitochondrial suspension was treated as the homogenate samples and stored in liquid nitrogen for determination of CoA and carnitine, or at −20°C for enzyme and protein assays.

All the measurements were made simultaneously on the tissue homogenates and on the homologous mitochondrial fractions. Acid-soluble CoA and carnitine, which includes both free and acetyl esters, were determined on the perchloric acid extract after alkaline hydrolysis, and the long-chain acyl esters were determined on the perchloric acid precipitate. Acid-soluble CoA and long-chain acyl-CoA were assayed fluorometrically using the α-ketoglutarate dehydrogenase reaction (Garland et al., 1965; Williamson and Corkey, 1969). Acid-soluble carnitine and long-chain acyl carnitine were assayed as free carnitine after hydrolysis by a radioisotope procedure (McGarry and Foster, 1976).

Mitochondrial marker enzymes were determined for the tissue homogenate and mitochondrial fractions. Citrate synthase activity was assayed according to the method of Srere (1969), and succinate dehydrogenase activity according to that of Singer and Kearney (1957). Protein concentration was determined by the Lowry procedure (1951) with bovine serum albumin as standard.

The aliquot of mitochondrial suspension used for electron microscopy was mixed with an equal volume of 2% glutaraldehyde in phosphate buffer, centrifuged, rinsed with buffer, and postfixed in 2% osmium tetroxide (Sordahl et al., 1971). The fixed mitochondrial pellets then were dehydrated and embedded in Epoxy resin. Thin sections were cut with a diamond knife and doubly stained with lead citrate and uranyl acetate before examination with the electron microscope.

**Results**

**Mitochondrial Content of Protein and Metabolites in Nonperfused Hearts**

For comparison of mitochondrial yield in the various groups of hearts, the cellular content of mitochondrial protein was estimated by measuring the activity of mitochondrial marker enzymes (citrate synthase and succinate dehydrogenase) in tissue homogenate and mitochondrial fractions. Mitochondrial content was calculated using the following formula:

\[
\text{mg of mitochondrial protein} = \frac{\text{mg of total protein}}{\text{g of muscle}} \times \frac{\text{homogenate S.A. of enzyme}}{\text{mitochondrial S.A. of enzyme}}
\]

as described by Idell-Wenger et al. (1978), where S.A. equals specific activity of enzyme in units/mg of total protein in the fraction. In nonperfused hearts, the yield of mitochondrial protein averaged 12 mg/g of wet tissue. The total mitochondrial protein calculated from the activities of the marker enzymes (Table 1) ranged between 46 and 58 mg/g of wet tissue for succinate dehydrogenase and citrate synthase, respectively.

Mitochondria isolated from ischemic hearts retained high levels of long-chain acyl carnitine and acyl-CoA only if they were isolated in the presence...
TABLE 1  Enzyme Activities and Protein Content in Nonperfused Hearts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Homogenate</th>
<th>Mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate synthase activity</td>
<td>0.68 ± 0.03</td>
<td>1.51 ± 0.04</td>
</tr>
<tr>
<td>(unit/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.069 ± 0.001</td>
<td>0.198 ± 0.010</td>
</tr>
<tr>
<td>activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(unit/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein content</td>
<td>134 ± 9</td>
<td>0.096 ± 0.003</td>
</tr>
<tr>
<td>(mg/g wet tissue)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg/mg total protein)</td>
</tr>
</tbody>
</table>

One unit of enzyme activity equals 1 µmol of substrate utilized or product formed per minute. Protein content is expressed as mg of total homogenate protein/g of wet tissue, or as mg of mitochondrial protein/mg of total protein of the homogenate (mitochondrial yield). Values are mean ± SEM for eight determinations.

of a respiratory inhibitor such as cyanide to prevent oxidation of these esters (Idell-Wenger et al., 1978). Thus, it appeared necessary to use a respiratory inhibitor in the present experiments. For each group of hearts, mitochondria were isolated both in the presence and absence of 1 mM cyanide. The ratio of acid-soluble carnitine:acyl carnitine has been plotted against the acid-soluble CoA:acyl-CoA ratio (Fig. 1). In the presence of cyanide (Fig. 1A) there is a good linear relationship (r = 0.87) between these ratios. However, when mitochondria were isolated in the absence of a respiratory inhibitor, there was no correlation between these ratios (Fig. 1B). These data indicate that oxidation of acyl derivatives occurs during the isolation of mitochondria in the absence of cyanide and that the levels found in the isolated mitochondria do not necessarily reflect that present in the intact tissue prior to homogenization. Therefore, only the results obtained from mitochondria isolated with cyanide are presented in subsequent tables and figures.

The cellular distribution of CoA and carnitine in nonperfused hearts is shown in Table 2. CoA and carnitine assays were done on aliquots of the homogenate and mitochondrial fractions that were analyzed for marker enzyme activities. Values for total tissue CoA and carnitine were calculated as the sum of acid-soluble and acid-insoluble fractions. The total CoA and carnitine in the mitochondria of the nonperfused hearts, calculated from the above formula, were, respectively, 93% and 6.5%.

Effects of Substrate and Ischemia on Mitochondrial CoA and Carnitine Levels

The ischemic perfusion conditions used in these experiments resulted in increased tissue levels of acyl esters of CoA and carnitine (Feuvray, 1981). The rise in tissue long-chain acyl carnitine was greater in hearts receiving palmitate than in those receiving no palmitate.

Mitochondrial levels of long-chain acyl-CoA and acyl carnitine in hearts perfused under control or ischemic conditions are shown in Table 3. Acyl-CoA
levels were higher in mitochondria isolated from hearts receiving palmitate (Table 3). There was no significant difference between acyl-CoA levels in mitochondria isolated from control and ischemic hearts receiving the same substrate. On the other hand, ischemia resulted in marked changes in the levels of acyl carnitine (Table 3). Levels of acyl carnitine in the mitochondria isolated from ischemic hearts were twice those measured in the mitochondria isolated from control hearts. Moreover, levels of acyl carnitine were much greater in the mitochondria isolated from ischemic hearts receiving palmitate. The ratio of acid-soluble carnitine : long-chain acyl carnitine was decreased from 48 to 28 by ischemia in mitochondria isolated from hearts receiving no palmitate, and from 16 to 8 in mitochondria isolated from hearts receiving palmitate.

Specific activities of marker enzymes in the mitochondria isolated from control and ischemic hearts were essentially the same (Table 4). This would indicate that mitochondrial breakage or, more exactly, loss during the isolation procedure in ischemic hearts was not different from that in control hearts, both in the presence or absence of palmitate.

### Effects of Substrate and Ischemia on the Mitochondrial Structure

As previously stated (Jennings and Ganote, 1976), it is important to analyze the purity of mitochondrial preparations, and purity is determined best by electron microscopy. The purity and structure of mitochondrial pellets used for biochemical analysis are shown in Figure 2. The vast majority of the mitochondria were well preserved (Fig. 2A). Relatively few disrupted mitochondria were present, and the pellets were devoid of contaminating cytoplasmic material.

Mitochondria isolated from both control and ischemic hearts receiving glucose as the only substrate (Fig. 2, B and C) did not show any obvious difference in structure. Under both conditions, some mitochondria had irregular, closely packed cristae, and a relatively dense matrix, while others exhibited a parallel array of cristae. Most mitochondria had a clear lucent matrix. Closely apposed membranes frequently were observed in ischemic mitochondrial preparations as well as in controls. This is a feature commonly encountered in isolated mitochondria and appeared to be nonspecific (Bullock et al., 1970).

### Table 2 Cellular Distribution of CoA and Carnitine in Nonperfused Hearts

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>Acid-soluble CoA (nM/mg protein)</th>
<th>Long-chain acyl-CoA (nM/mg protein)</th>
<th>Acid-soluble carnitine (nmol/mg protein)</th>
<th>Long-chain acyl carnitine (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.970 ± 0.057</td>
<td>0.326 ± 0.018</td>
<td>20.72 ± 0.42</td>
<td>0.942 ± 0.055</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>2.06 ± 0.03</td>
<td>0.67 ± 0.03</td>
<td>3.60 ± 0.06</td>
<td>0.230 ± 0.012</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for 12 hearts.

### Table 3 Effects of Substrate and Ischemia on Mitochondrial Levels of CoA, Carnitine, and Their Long-chain Acyl Esters, in Perfused Hearts

<table>
<thead>
<tr>
<th>Perfusion condition</th>
<th>Acid-soluble CoA (nmol/mg protein)</th>
<th>Long-chain acyl-CoA (nmol/mg protein)</th>
<th>Acid-soluble carnitine (nmol/mg protein)</th>
<th>Long-chain acyl carnitine (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.14 ± 0.08</td>
<td>0.35 ± 0.04</td>
<td>3.33 ± 0.28</td>
<td>0.066 ± 0.003</td>
</tr>
<tr>
<td>Ischemia</td>
<td>1.20 ± 0.05</td>
<td>0.49 ± 0.04</td>
<td>3.88 ± 0.28</td>
<td>0.136 ± 0.021</td>
</tr>
<tr>
<td>Glucose plus palmitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.99 ± 0.09</td>
<td>0.87 ± 0.11</td>
<td>4.22 ± 0.57</td>
<td>0.259 ± 0.030</td>
</tr>
<tr>
<td>Ischemia</td>
<td>0.76 ± 0.11</td>
<td>1.03 ± 0.08</td>
<td>4.10 ± 0.43</td>
<td>0.498 ± 0.047</td>
</tr>
</tbody>
</table>

The values represent the mean ± SEM for five to seven hearts.

### Table 4 Enzyme Activities in Mitochondria Isolated from Perfused Hearts

<table>
<thead>
<tr>
<th>Perfusion condition</th>
<th>Citrate synthase (U/mg protein)</th>
<th>Succinate dehydrogenase (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Homogenate</td>
<td>0.75 ± 0.01</td>
<td>0.064 ± 0.020</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.45 ± 0.04</td>
<td>0.143 ± 0.009</td>
</tr>
<tr>
<td>Ischemia Homogenate</td>
<td>0.69 ± 0.01</td>
<td>0.061 ± 0.030</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.49 ± 0.02</td>
<td>0.121 ± 0.009</td>
</tr>
<tr>
<td>Glucose plus palmitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Homogenate</td>
<td>0.78 ± 0.02</td>
<td>0.072 ± 0.004</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.45 ± 0.02</td>
<td>0.152 ± 0.003</td>
</tr>
<tr>
<td>Ischemia Homogenate</td>
<td>0.75 ± 0.01</td>
<td>0.069 ± 0.006</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.47 ± 0.07</td>
<td>0.211 ± 0.011</td>
</tr>
</tbody>
</table>

Perfusion conditions were the same as for Table 3. Values are mean ± SEM for five to seven hearts.
FIGURE 2   Micrographs of pellets of mitochondria. Mitochondria were isolated in MSE buffer by the polytron technique. A: Mitochondria of nonperfused heart. Numerous intact mitochondria and some inner membrane fragments are present. Note the absence of cytoplasmic contaminants (6000X). B: Mitochondria of control heart perfused for 65 minutes with glucose alone as substrate. Note the frequent close apposition of inner membranes that are more rarely seen in mitochondria isolated from nonperfused hearts (6000X). C: Mitochondria of heart perfused 60 minutes under ischemic condition with glucose alone as the substrate. No noticeable difference could be observed, compared to B, but numerous close appositions of mitochondrial inner membranes (arrow) are observed, as illustrated by this high-powered view (40000X).

The structure of mitochondria isolated from hearts receiving palmitate is shown in Figure 3. There appears to be a higher proportion of damaged mitochondria in these ischemic hearts (Fig. 3B) compared to those from control hearts (Fig. 3A). Numerous intramitochondrial dense staining bodies were observed. This dense staining material (Fig. 4, A and B) looked very similar to the amorphous densities seen in the intact tissue under comparable situations (Feuvray, in press). Moreover, these amorphous densities were seen predominantly in those mitochondria isolated in the presence of a respiratory inhibitor and were associated with high levels of acyl-CoA and acyl carnitine. Under high magnification, it appeared that the densities were either located in folds of the inner membrane or that the inner membranes were fused at places where the densities occurred. This fusion could alter the membrane in such a way as to give the appearance of an amorphous density.

Discussion

Development of intramitochondrial amorphous densities occurs in many forms of severe cell injury (Reimer et al., 1972; Ganote et al., 1974). These densities have been particularly well described in severe myocardial ischemic injury (Jennings et al., 1965; Jennings and Ganote, 1972, 1976; Trump et al., 1976; de Leiris and Feuvray, 1977; Schaper et al., 1979), and their appearance is a characteristic feature of the irreversibility of damage in blood perfused hearts.

Most of the above studies used intact tissues. Jennings et al. (1969) and Jennings and Ganote (1976) isolated mitochondria by differential centrifugation. However, this procedure yields only a fraction of the total mitochondrial population, and in the case of ischemic tissue, the severely damaged mitochondria may be lost during the isolation. Electron microscopy of the mitochondria that are isolated with the polytron technique attested to the purity and, also, to the good preservation of these organelles. Palmer et al. (1977) have described two populations of mitochondria that could be more or less easily released depending on the isolation procedure used. In the present experiments, however, it appears that damaged mitochondria were recovered, at least from those ischemic hearts receiving palmitate. Damaged mitochondria containing amorphous densities were located mainly near the sarcolemma, the intercalated discs, or in the vicinity of damaged myofibrils and were easily released from the ischemic tissue (Feuvray, in press).

We did not observe any difference in mitochondria isolated from control and ischemic hearts receiving glucose. Mitochondrial morphology is af-
Figure 3  Micrographs of pellets of mitochondria isolated from perfused hearts receiving 1.5 mM palmitate. A: After 60 minutes under control perfusion, mitochondria appear very similar to those observed in Figure 2B (10,750x). B: After 60 minutes under ischemic condition, numerous damaged mitochondria are seen. Moreover, many dense staining areas (amorphous densities) are present (arrows) in both the more or less damaged mitochondria (10,750x).

affected only slightly in the ischemic tissue in the presence of glucose alone, and marked structural differences must occur before they can be detected by electron microscopy of the isolated organelles.

Biochemical determinations were made on poltron-sonicated mitochondria. The specific marker enzyme activities were about the same in mitochondria isolated from ischemic and from normal hearts. This occurred even in the mitochondria isolated from ischemic hearts receiving palmitate where structural intactness was lost but apparently the enzymes were not. Increased tissue levels of both long-chain acyl-CoA and acyl carnitine occur during myocardial ischemia (Whitmer et al., 1978; Feuvray, 1979). After long-term ischemia in hearts receiving palmitate, there was a good correlation between the development of a large number of intramitochondrial amorphous densities and the great increase in the tissue level of acyl carnitine (Feuvray, in press). In the present study, there was also an association between the rise in acyl carnitine levels and structural alterations in mitochondria isolated from ischemic tissue.

Our data indicate clearly that a higher level of long-chain fatty acyl carnitine was associated with the mitochondria isolated from ischemic hearts receiving a high level of fatty acid. These data are in agreement with those of Idell-Wenger et al. (1978) who found that an increased portion of the cellular long-chain acyl carnitine was associated with the mitochondria isolated from ischemic tissue receiving glucose plus palmitate. As in our study, these results were particularly evident when mitochondria were isolated in the presence of cyanide to prevent the oxidation of long-chain acyl derivatives. Wood et al. (1973) reported specific changes in the fatty acid transferase system of mitochondria from ischemic hearts. The irreversibility of this defect may be due, at least in part, to structural changes in the mitochondrial membrane lipid.

Our present data, as well as previous work (de Leiris and Feuvray, 1977; Mochizuki et al., 1980; Feuvray, in press), suggest that upon exposure of tissue to excess fatty acids, acyl carnitine may accumulate on the mitochondrial membrane and cause structural alterations. These alterations may be due in part to the detergent effect of acyl carnitine. In the present study, alterations are visible under the electron microscope as amorphous densities. In isolated mitochondria, the amorphous densi-
Figure 4  A: Mitochondria isolated from an ischemic heart receiving palmitate. An amorphous density (arrow) is present in this area of diffuse material (43,600×). B: Mitochondria containing amorphous densities (arrows) in the ischemic tissue (heart perfused in similar conditions) is shown for comparison (41,600×).

Amorphous densities appear to be bound to the cytosolic side of the inner membrane as if altered membranes had fused (Fig. 4A). Since amorphous densities were found only in hearts receiving fatty acid, the present data support the previous suggestions of Jennings and Ganote (1976) that these amorphous osmiophilic densities are lipid in nature. They may, however, represent the rearrangement of lipids from mitochondrial membranes due to the combined detergent action of acyl-CoA and acyl carnitine.

Although amorphous densities are characteristic of irreversibly injured myocardium (Jennings and Ganote, 1976) there are no data to support a causal relationship. Most previous studies in this area have used blood-perfused hearts in which fatty acids are always present. Thus a cause-and-effect relationship could not be determined. The present data suggest that use of the isolated heart, in which the development of amorphous densities is related to the supply of fatty acids, may allow the relationship between the morphological changes in mitochondria and the development of irreversible injury to be determined in future studies.

Acknowledgments

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