Reversal of Ischemic Mitochondrial Dysfunction*

(Received for publication, December 11, 1978)

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Lever ischemia in intact rats is associated with a series of alterations in mitochondrial structure and function that include: a complete loss of respiratory control; a loss of adenine nucleotide translocase activity; decreases in, at least, the heme portions of cytochromes aa₃ and c + c₁; a decrease in dinitrophenol-activated ATPase; a loss of the ability of dinitrophenol to stimulate O₂ uptake; a decrease in the content of one Mr = 83,000 protein band; and lastly, changes in mitochondrial ultrastructure characterized by swelling, loss of a tightly folded and contorted inner membrane, and the appearance of amorphous matrix densities. After 3 h of ischemia, none of these alterations are restored upon reestabilishment of liver blood flow. An identical sequence of mitochondrial alterations occurs in ischemic liver tissue that has been pretreated with chlorpromazine. However, in the chlorpromazine-treated animals all of these mitochondrial alterations are completely reversible even after 3 h of ischemia. The inability to restore mitochondrial function during reperfusion in the absence of chlorpromazine, therefore, cannot be the direct consequence of any of these alterations. Rather, it would seem to be the metabolic consequence of reperfusion itself. In the same way, these mitochondrial alterations cannot be the cause of the irreversibility of the cellular deterioration and death during the reperfusion period. The mechanisms for the effects of ischemia on mitochondrial structure and function and the ability to reverse these changes in the presence of chlorpromazine are discussed.

The role of mitochondrial damage in the pathogenesis of irreversible, ischemic cell injury has been a matter of debate for some time. In vitro studies of kidney, liver, heart, and brain as well as of several in vitro models have documented that an inability to reverse mitochondrial dysfunction upon reperfusion or reoxygenation correlated with a similar inability to reverse the cell injury in general (1-7). Such studies have been interpreted as suggesting that ischemic cell death is a consequence of irreversible mitochondrial injury (6, 7). Reperfusion, however, may expose the injured cells to an environment that does not allow recovery of mitochondrial function. In particular, it has been shown that during reperfusion of irreversibly injured cells a large influx of Ca²⁺ ions occurs (8, 9). High levels of Ca²⁺ are known to result in the loss of mitochondrial function (10). It is conceivable that an inability to reverse mitochondrial dysfunction may be the consequence of the flooding of the cell with Ca²⁺ rather than the consequence per se of the specific pattern of mitochondrial dysfunction prior to reflow. A critical test, therefore, of the role of mitochondrial dysfunction in the pathogenesis of irreversible ischemic cell injury must include the prevention of Ca²⁺ accumulation by these organelles.

We have developed a system with which to assess the reversibility of ischemic mitochondrial dysfunction in the absence of such Ca²⁺ accumulation. Liver ischemia in intact rats is accompanied by a progressive depletion of cellular phospholipids associated with manifestations of microsomal and plasma membrane dysfunction (11). Upon reflow such irreversibly injured cells accumulate large quantities of Ca²⁺ with significant rises in mitochondrial Ca²⁺ content (7). Pretreatment of the rats with the phenothiazine chlorpromazine prevents the accelerated degradation of phospholipids without altering the rate or extent of the liver ischemia (9). In the present report we show that chlorpromazine pretreatment does not alter the pattern of mitochondrial alterations associated with liver ischemia. In the absence of chlorpromazine mitochondrial function cannot be restored (9). In its presence, however, a complete loss of respiratory control secondary to inhibition of adenine nucleotide translocator, a loss of mitochondrial cytochromes, and changes in protein composition and in ultrastructure are completely reversible. These mitochondrial alterations are, therefore, unlikely to be a cause of irreversible injury. The failure to reverse similar changes in previous studies is most likely the consequence of the metabolic alterations that accompany reperfusion of cells irreversibly injured by a mechanism unrelated to the loss of mitochondrial structure and function.

MATERIALS AND METHODS

Female Wistar rats (Charles River Breeders) weighing 140 to 165 g were fasted 18 h overnight prior to use. Liver ischemia in the intact rat was induced under ether anesthesia by placing a small arterial clamp across the hepatic arterial and portal venous blood supply to the left lateral and median lobes as described previously (9). The laparotomy wound was stapled shut. The animal regained consciousness within minutes and was free to move about during the remainder of the period of liver ischemia. In order to establish reperfusion of the ischemic lobes, the animal was anesthetized again, the abdominal wound was reopened, and the arterial clamp was removed. After closing the abdominal wound, the animal was again free to move about for the remainder of the experiment. Chlorpromazine (Smith, Kline and French) was administered by intraperitoneal injection of a 10 mg/ml solution at a dose of 30 mg/kg 30 min before surgery. Animals were killed by decapitation and mitochondria were isolated by the method of Schneider and Hogeboom (12) in 0.25 M sucrose containing 1 mM EDTA, pH 7.4. and 1 mg/ml of bovine serum albumin. The mitochondria were suspended in the 0.25 M sucrose solution at 15 to 25 mg of protein/ml. Protein was determined by the method of Lowry et al. (13).

Measurement of Respiratory Control—The respiratory control and ADP:O ratios were determined as described by Estabrook (14). The reaction mixture contained 0.295 M sucrose, 10 mM potassium

The Journal of Biological Chemistry
Vol. 254, No. 19, Issue of October 10, pp. 9871-9878, 1979
Printed in U.S.A.
phosphate (pH 7.4), 5 mM MgCl₂, 20 mM KCl, and 20 mM triethanolamine buffer, pH 7.4. The oxidizable substrate was either 3.3 mM succinate or 3.3 mM glutamate, 3.3 mM malate. After equilibration of the medium at 25°C mitochondria (2 to 4 mg of protein) were added and the rate of O₂ consumption was measured with a YSI O₂ monitor (Yellow Springs Instruments, Yellow Springs, Ohio) equipped with a Clark-type electrode and a regulated water bath. State 3 respiration was initiated by adding 450 or 900 nmol of ADP. Uncoupled respiration was measured in the presence of 33 μM dinitrophenol.

Measurement of Enzyme Activities—Cytochrome oxidase was measured by the rate of oxidation of ferrocyanochrome c as determined by the decrease in absorbance of its a-band at 550 nm. Mitochondria were solubilized with 0.2 mg/ml in 0.25 M sucrose, 1 mM EDTA, pH 7.4, containing 1 mg/ml of bovine serum albumin and 0.1% Triton X-100. The reaction was initiated by adding 10 μl of diluted mitochondria to the sample cuvette containing 2 ml of 0.1 M potassium phosphate, pH 6.0, and various concentrations of reduced cytochrome c. The reference cuvette contained 2 ml of 0.1 M potassium phosphate, equivalent concentrations of cytochrome c, and 0.100 ml of 50 mM potassium ferricyanide. A stock solution of 5 ml of 1 mM cytochrome c (Sigma) in 10 M potassium phosphate, pH 7.4, and 1 mM EDTA was reduced with L-ascorbate (Fisher) and dialyzed for 24 h at 4°C against 10 M potassium phosphate, 1 mM EDTA. This preparation remains 96 to 99% reduced up to several months.

Mitochondrial ATPase was determined by measuring the production of inorganic phosphate from 6 μM ATP in a 2 ml reaction mixture containing intact mitochondria (2 to 3 mg of protein), 74 mM KCl, 50 mM sucrose, 50 mM Tris-HCl (pH 7.4), and 1 mM EDTA. MgCl₂, and dinitrophenol when present were 4 and 1 mM, respectively. For some experiments mitochondria were sonicated for a total of 15 s with a Heat Systems model W 185 sonifier with an output setting of 6 (50 to 60 watts). The incubation was at 55°C for 15 min in a shaking water bath. The reaction was terminated by the addition of 0.5 ml of ice-cold 10% trichloroacetic acid and the samples were immediately centrifuged at top speed in a clinical tabletop centrifuge for 10 min. A 0.25-ml aliquot of the supernatant was assayed for inorganic phosphate by the Fiske and Subbarow method (13).

Measurement of Adenine Nucleotide Translocase—Adenine nucleotide translocase was measured by modification of the method of Vignais et al. (17) with ATP substituted for ADP. Mitochondria (approximately 1 mg of protein) were added to 1 ml of a solution containing 110 mM KCl, pH 7.4, 0.2 mM EDTA, and 70 mM of [14C]ATP (3 to 5 × 10⁷ cpm). After incubation at 4°C, the reaction was stopped by the addition of 50 mM of atracrylate (Sigma). The samples were centrifuged within 2 min of the addition of atracrylate for 10 min at 10,000 × g. The supernatant was discarded and the pellet was dissolved in 150 μl of 0.5% sodium dodecyl sulfate. A 100-μl aliquot of the dissolved mitochondrial pellet was counted in 10 ml of Aquasol (New England Nuclear).

Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis of solubilized mitochondrial proteins was performed on 15-cm gels according to the method of Summers et al. (18). Mitochondrial proteins were solubilized in 100 to 150 μl of mercaptoethanol and 1 to 2 ml of 0.1% sodium dodecyl sulfate in 10 M Tris-HCl, pH 7.4, for 5 min at 90°C. The gels were prerun for 30 min at 25 V, run at 25 V for 30 min, and then run at 50 V for 18 h.

Electron Microscopy of Normal and Ischemic Mitochondria—Mitochondrial pellets were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.2, for 20 min on ice. The pellet then was cut into small pieces and fixed for an additional hour. The pellets were washed for 30 min in the 0.1 M phosphate buffer and postfixed for 1 h with 1% OsO₄ in the same buffer. After dehydration with ethanol, the samples were infiltrated with and embedded in eponaraldite mixture. Sections were cut on a Sorvall Porter-Blum MT-2 microtome with a diamond knife on a Phillips EM300 operating at 80 kV. Photographs were taken on Kodak electron image plates.

RESULTS

Respiratory Control in Ischemic Mitochondria—Mitochondria were isolated from rat liver made ischemic in the intact animal and were assayed for respiratory control with glutamate/malate as substrate. In Fig. 1 such mitochondria show a progressive loss of respiratory control, the respiratory control ratio (RCR) falling to the theoretical limit of 1 with 3 h of ischemia. After 2 or 3 h of ischemia the livers were reperfused with blood by re-operating on the animals and removing the arterial clamp occluding the arterial and portal blood supply to the left lateral and median lobes. — —, mitochondria prepared subsequent to reperfusion; — —, mitochondria prepared from ischemic livers without reperfusion. Mitochondria were isolated and the respiratory control ratio was measured with glutamate/malate as substrate as described under "Materials and Methods." The results are the mean ± standard deviation of separate preparations from each of three animals.

If the rats are pretreated with chlorpromazine and then liver ischemia is induced for the same times, there is a similar loss of respiratory control (Fig. 1, open circles). With chlorpromazine pretreatment the RCR still falls to 1 with 3 h of ischemia. In this case, however, reperfusion of the livers after either 2 or 3 h of ischemia results in complete recovery of respiratory control (Fig. 1, dashed line with closed circles). This is consistent with the fact that the liver cells are reversibly injured after such ischemic periods (19, 20), and it is such a correlation between the loss of reversibility and the inability to reverse mitochondrial dysfunction that has led to the suggestion that the two are causally related.

1 The abbreviations used are: RCR, respiratory control ratio; DNP, dinitrophenol.
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TABLE I
State 3 and State 4 respiratory rates in ischemic liver mitochondria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>State 4*</th>
<th>State 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol O_2/mg protein/min</td>
<td></td>
</tr>
<tr>
<td>1. Sham-operated</td>
<td>20 ± 3.2</td>
<td>84 ± 4.2</td>
</tr>
<tr>
<td>2. Chlorpromazine pretreatment, sham-operated</td>
<td>16 ± 1.7</td>
<td>71 ± 3.8</td>
</tr>
<tr>
<td>3. Ischemia, 3 h</td>
<td>14 ± 1.3</td>
<td>14 ± 1.3</td>
</tr>
<tr>
<td>4. Ischemia, 3 h, 2 h reflow</td>
<td>12 ± 2.6</td>
<td>12 ± 2.6</td>
</tr>
<tr>
<td>5. Chlorpromazine pretreatment, 3 h ischemia</td>
<td>15 ± 2.2</td>
<td>15 ± 2.2</td>
</tr>
<tr>
<td>6. Chlorpromazine pretreatment, 3 h ischemia, 2 h reflow</td>
<td>18 ± 2.1</td>
<td>73 ± 4.2</td>
</tr>
</tbody>
</table>

* Results are the mean ± standard deviation of determinations on each of three separate preparations.

TABLE II
Respiratory control with succinate as substrate in ischemic liver mitochondria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Respiratory control ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham-operated</td>
<td>5.5 ± 0.44</td>
</tr>
<tr>
<td>2. Chlorpromazine pretreatment, sham-operated</td>
<td>4.9 ± 0.47</td>
</tr>
<tr>
<td>3. Ischemia, 3 h</td>
<td>1.5 ± 0.20</td>
</tr>
<tr>
<td>4. Ischemia, 3 h, 2 h reflow</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>5. Chlorpromazine pretreatment, 3 h ischemia</td>
<td>2.0 ± 0.17</td>
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<tr>
<td>6. Chlorpromazine pretreatment, 3 h ischemia, 2 h reflow</td>
<td>4.6 ± 0.32</td>
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* Results are the mean ± standard deviation of separate determinations on mitochondria isolated from three animals.

Table III
ADP:O ratios in ischemic liver mitochondria

<table>
<thead>
<tr>
<th>Treatment</th>
<th>With chlorpromazine pretreatment</th>
<th>Without chlorpromazine pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sham-operated</td>
<td>2.38 ± 0.22</td>
<td>2.45 ± 0.32</td>
</tr>
<tr>
<td>2. Ischemia, 0.5 h</td>
<td>2.49 ± 0.08</td>
<td>2.41 ± 0.32</td>
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<tr>
<td>3. Ischemia, 1 h</td>
<td>2.46 ± 0.15</td>
<td>2.50 ± 0.20</td>
</tr>
<tr>
<td>4. Ischemia, 2 h</td>
<td>1.88 ± 0.31</td>
<td>1.95 ± 0.05</td>
</tr>
<tr>
<td>5. Ischemia, 3 h, 2 h reflow</td>
<td>2.64 ± 0.07</td>
<td></td>
</tr>
</tbody>
</table>

* Results are the mean ± standard deviation of separate determinations on each of three preparations of mitochondria.

The peak at 551 nm is cytochrome c + c1. The content of these cytochromes calculated from this spectrum according to the method of Williams (21) is similar to that reported previously (22). In mitochondria isolated from livers made ischemic for 3 h there was a fall in the contents of cytochrome aa3 and c + c1 (Fig. 2, Tracing b). A similar decrease in cytochromes aa3 and c + c1 occurred in ischemic livers from animals pretreated with chlorpromazine (Fig. 2, Tracing c). Tracing d in Fig. 2 shows the cytochromes in mitochondria obtained after 3 h of ischemia and 2 h of reperfusion in a chlorpromazine-pretreated animal. Both cytochrome aa3 and c + c1 have been restored to control levels. In contrast, there was no recovery of cytochrome content after a similar period of reperfusion in the absence of chlorpromazine pretreatment (Fig. 2, Tracing e). These changes in cytochrome contents were confirmed by the direct analysis of extracted cytochrome hemes (data not shown), indicating that the reduced cytochrome contents in Fig. 2 cannot be explained simply as a

The ADP:O ratio was also decreased in ischemic mitochondria (Table III). The ADP:O ratio was not changed after 1 h of ischemia. With 2 h ischemia in the presence or absence of chlorpromazine there was a 20 to 25% decrease in the ADP:O ratio (Table III). This contrasts with a greater than 60% decrease in the RCR at the same time (Fig. 1).

In all cases the yield of mitochondria from ischemic livers was similar to that from the controls. The phospholipid/protein ratio (micromoles of PO_4/mg of protein) of mitochondria isolated from ischemic livers either with (0.25 ± 0.007) or without chlorpromazine pretreatment (0.23 ± 0.016) was the same as that from sham-operated animals (0.23 ± 0.007).

The Electron Transport Chain in Ischemic Mitochondria—The inability of ADP to stimulate O_2 uptake in ischemic mitochondria cannot be explained by a loss of the capacity of metabolizing glutamate, malate, or succinate. The specific activities of malate dehydrogenase, glutamate/oxaloacetate transaminase, and succinic dehydrogenase were the same in mitochondria isolated from sham-operated animals or from livers made ischemic for 3 h with or without chlorpromazine pretreatment.

Ischemia did, however, produce significant changes in the contents of the electron transport cytochromes. Fig. 2 shows representative absorption difference spectra (ferricyanide-oxidized versus dithionite-reduced) of mitochondria solubilized in Triton X-100 and scanned between 650 and 500 nm, the region containing the a-bands of the cytochromes. The mitochondrial cytochromes from sham-operated animals are shown in Fig. 2, Tracing a. The peak of absorption at 565 nm is cytochrome aa3, the peak at 562 nm is cytochrome b, and the peak at 551 nm is cytochrome c + c1. The content of these cytochromes calculated from this spectrum according to the method of Williams (21) is similar to that reported previously (22). In mitochondria isolated from livers made ischemic for 3 h there was a fall in the contents of cytochrome aa3 and c + c1 (Fig. 2, Tracing b). A similar decrease in cytochromes aa3 and c + c1 occurred in ischemic livers from animals pretreated with chlorpromazine (Fig. 2, Tracing c). Tracing d in Fig. 2 shows the cytochromes in mitochondria obtained after 3 h of ischemia and 2 h of reperfusion in a chlorpromazine-pretreated animal. Both cytochrome aa3 and c + c1 have been restored to control levels. In contrast, there was no recovery of cytochrome content after a similar period of reperfusion in the absence of chlorpromazine pretreatment (Fig. 2, Tracing e). These changes in cytochrome contents were confirmed by the direct analysis of extracted cytochrome hemes (data not shown), indicating that the reduced cytochrome contents in Fig. 2 cannot be explained simply as a
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3. While an obvious feature of the reaction of mitochondria to ischemia, these changes in the content of the electron transfer cytochromes were not accompanied by any loss of the activity of the enzymes mediating electron transport. Representative of the data supporting this conclusion is that shown in Table IV. The rate of oxidation of added, reduced cytochrome c by cytochrome oxidase was measured in Triton X-100-solubilized mitochondria from sham-operated and 3-h ischemic mitochondria. There was no change in the calculated maximal activity of cytochrome oxidase expressed as micromoles of cytochrome c oxidized/min/mg of mitochondrial protein nor in the concentration of cytochrome c that gave half-maximal activity (Table IV). These data would also indicate that the loss of respiratory control cannot be explained by the presence of an inhibitor of electron transport.

Adenine Nucleotide Translocase and ATPase Activity in Ischemic Mitochondria—The ability both to transport adenine nucleotides across the inner mitochondrial membrane and subsequently to synthesize ATP can be evaluated by measurement of mitochondrial ATPase activity. A loss of ATPase activity could reflect a disturbance in the adenine nucleotide translocator or in the ATP synthetase complex, or both. Control liver mitochondria show a low level of ATPase in the absence of either Mg$^{2+}$ ions or dinitrophenol (Table V). Mg$^{2+}$ ions alone increase this basal level some 3-fold. DNP alone increases the ATPase activity some 60-fold and there is no additional effect of Mg$^{2+}$ in the presence of DNP. Ischemia produced two alterations in these ATPase activities (Table V). The basal activity in the absence of either Mg$^{2+}$ or DNP is some 3- to 4-fold higher than in control mitochondria. Second, DNP alone produced much less stimulation of the basal ATPase, resulting in a 4-fold lower activity with DNP and Mg$^{2+}$ ions. This pattern of alteration of ATPase activity was seen with or without chlorpromazine pretreatment, and there was no change with reflow in the absence of chlorpromazine. However, there was recovery of a normal pattern of ATPase activity with reflow to the chlorpromazine-pretreated ischemic livers (Table V).

4. After 2 h of reflow it is evident there is a decline in the ATPase activity with Mg$^{2+}$ alone and an increase in the stimulation by DNP to essentially control levels. The loss of DNP-activated ATPase in ischemic mitochondria was paralleled by a similar inability of DNP to increase the rate of O$_2$ consumption in sham-operated mitochondria from 20 (Table I) to 76 nmol/mg of protein/min (Table VI). In contrast, DNP had only a very slight effect on the rate of O$_2$ uptake in mitochondria isolated from ischemic livers with or without chlorpromazine pretreatment (Table VI). Two hours of reflow completely restored the control rate of DNP-induced O$_2$ uptake in mitochondria from chlorpromazine-treated animals. There was no effect of reflow on DNP-induced O$_2$ uptake in the absence of chlorpromazine pretreatment (Table VI).

Both the demonstration of respiratory control by added ADP and the measurement of ATPase with added ATP are dependent upon transport of these adenine nucleotides across the mitochondrial inner membrane. Loss of adenine nucleotide translocase activity has been reported in ischemic heart muscle (23-25). Fig. 3 shows that 3 h of ischemia produced a very significant loss of adenine nucleotide translocase in ischemic liver mitochondria. During a 10-min incubation at 4°C ischemic mitochondria accumulated 85% less [14C]ATP than control mitochondria. Fig. 3 also shows that a similar loss of adenine nucleotide translocase occurred in the mitochondria from 3-h ischemic animals pretreated with chlorpromazine. Recovery of adenine nucleotide translocase activity with blood reflow following 3 h of ischemia was achieved in animals pretreated with chlorpromazine (Fig. 3). There was no recovery of adenine nucleotide translocase activity with reflow to the chlorpromazine-pretreated ischemic livers (Fig. 3).

### Table IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol oxidized/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-operated</td>
<td>22.0 ± 5.25</td>
<td>12.2 ± 0.60</td>
</tr>
<tr>
<td>Ischemia, 3 h</td>
<td>33.4 ± 6.39</td>
<td>11.8 ± 1.66</td>
</tr>
</tbody>
</table>

*Results are the mean ± standard deviation of separate determinations on each of three mitochondrial preparations.

### Table V

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATPase activities in ischemic liver mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Additions to assay</td>
</tr>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>μmol ATP/mg protein/h</td>
</tr>
<tr>
<td>1. Sham-operated</td>
<td>0.3 ± 0.00</td>
</tr>
<tr>
<td>2. Ischemia, 2 h</td>
<td>1.4 ± 0.21</td>
</tr>
<tr>
<td>3. Ischemia, 3 h, 2 h reflow</td>
<td>1.2 ± 0.25</td>
</tr>
<tr>
<td>4. Chlorpromazine, 3 h ischemia</td>
<td>0.8 ± 0.14</td>
</tr>
<tr>
<td>5. Chlorpromazine, 3 h ischemia, 2 h reflow</td>
<td>0.5 ± 0.06</td>
</tr>
<tr>
<td>6. Control mitochondria + 50 μM atracyloside</td>
<td>0.3 ± 0.23</td>
</tr>
<tr>
<td>7. Control mitochondria + 50 μM atracyloside + sonication</td>
<td>1.3 ± 0.71</td>
</tr>
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</table>

*Results are the mean ± standard deviation of separate determinations on each of three mitochondrial preparations.
A strong correlation exists between the extent of inhibition of adenine nucleotide translocase as assessed by the rate of uptake of [14C]ATP and the respiratory control ratio as assessed by the increase in O2 consumption with addition of ADP. In Fig. 4 representative values of the 10-min specific activity of adenine nucleotide translocase in mitochondria obtained from animals made ischemic for various periods of time are plotted against the respiratory control ratio of the same mitochondria. The calculated correlation coefficient between the two sets of data is 0.91, implying that loss of adenine nucleotide translocase activity can account for the loss of respiratory control in ischemic mitochondria.

In order to determine whether loss of adenine nucleotide translocase could also explain the loss of ATPase activity and DNP-induced O2 uptake, the effect of atractyloside on liver mitochondria in vitro was used to model the effects...
of ischemia on mitochondria in vivo. Addition of 50 μM atractyloside to control mitochondria inhibited DNP-stimulated ATPase activity without increasing the basal activity (Table V). Atractyloside did not prevent, however, the DNP-induced increase in O₂ uptake in control mitochondria. Sonication of control mitochondria treated with atractyloside restored Mg²⁺ ATPase activity. Sonication of ischemic mitochondria has no effect on Mg²⁺ ATPase activity.

Structure of Ischemic Mitochondria—Fig. 5 illustrates the electrophoretic profiles of mitochondrial proteins in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 0.5 mM urea. The profiles are complex in keeping with the large number of polypeptides present in mitochondria. Nevertheless, a constant pattern was obtained in all preparations, with one significant difference between the proteins in normal and ischemic mitochondria. One band which migrates in the region of Mr = 83,000 is reduced markedly after 3 h of ischemia (Fig. 5, Tracing b). Chlorpromazine pretreatment did not prevent the decrease in this band (Fig. 5, Tracing c). In the chlorpromazine pretreated animals, the amount of this band was restored with 2 h of reperfusion (Fig. 5, Tracing d), while without chlorpromazine there was no recovery (Fig. 5, Tracing d).

Discussion

The present report describes a number of alterations in the structure and function of rat liver mitochondria that develop with ischemia and include: a complete loss of respiratory control (Fig. 1); a loss of adenine nucleotide translocase activity (Fig. 3); decreases in ATPase activity without increasing the heme portions of cytochromes aa₃ and c + c₁ (Fig. 2); a decrease in dinitrophenol-activated ATPase (Table V); a loss of the ability of DNP to stimulate O₂ uptake (Table VI); marked decreases in the content of one Mr = 83,000 protein band (Fig. 5); and lastly, changes in ultrastructure characterized by swelling, loss of a tightly folded and contorted inner membrane, and the appearance of amorphous matrix densities (Fig. 6). After 3 h of ischemia these alterations are not reversible with reperfusion of the livers. An identical sequence of mitochondrial alterations occurs in ischemic liver tissue that has been pretreated with chlorpromazine. Fig. 1 shows that mitochondria from chlorpromazine-treated animals completely lose respiratory control. Fig. 3 shows that there is a loss of adenine nucleotide translocase. Fig. 2 indicates that chlorpromazine does not prevent the loss of cytochrome aa₃ and c + c₁. Chlorpromazine does not prevent the loss of either DNP-activated ATPase (Table V) or DNP-stimulated O₂ uptake (Table VI). In Fig. 5 ischemic mitochondria from chlorpromazine-treated animals are depleted of the same protein band lost with ischemia alone. In Fig. 6 ischemic mitochondria from chlorpromazine-treated animals have the same ultrastructural appearance as ischemic mitochondria alone. In the presence of chlorpromazine all of these alterations were completely reversible. The lack of restoration of mitochondrial function during reperfusion in animals not pretreated with chlorpromazine, therefore, cannot be the direct consequence of any of these alterations. Rather, it would seem to be the metabolic consequence of the irreversibility of the cellular deterioration and death during the reperfusion period.

The results of the present study extend our understanding of the effects of ischemia on liver cells and of the action of chlorpromazine in preventing the ischemic death of these cells. We have previously documented that liver ischemia is associated with an accelerated degradation of phospholipids (11). Phospholipids are lost from the membranes of the endoplasmic reticulum (11) and most likely from the plasma membrane (31). Pretreatment of the animals with chlorpromazine prevents this phospholipid degradation and its
associated disturbances in microsomal and plasma membrane function (11, 31, 32). The alterations in mitochondrial structure and function described in the present report must result from metabolic disturbances unrelated to the accelerated phospholipid degradation. There is no loss of phospholipid from the ischemic mitochondria, and the mitochondrial alterations produced by ischemia are not prevented by chlorpromazine. However, the restoration of mitochondrial function during reperfusion probably relates to the prevention by chlorpromazine of the disorder in phospholipid metabolism. While 3 h of liver ischemia are not accompanied by any increases in mitochondrial Ca\(^{2+}\) content, reperfusion of the livers is associated with a more than 4-fold rise in the mitochondrial Ca\(^{2+}\) content (9). With chlorpromazine pretreatment reperfusion of ischemic livers is not accompanied by an increased mitochondrial Ca\(^{2+}\) content. Ischemic microsomal membranes have been shown to be some 25- to 50-fold more permeable to Ca\(^{2+}\) ions than are control membranes. A similar increase in Ca\(^{2+}\) permeability of the plasma membrane would produce an influx of Ca\(^{2+}\) ions into the lethally injured cells upon reperfusion. The increased mitochondrial Ca\(^{2+}\) content upon reperfusion then would be a consequence of this increased liver cell Ca\(^{2+}\) content. High levels of Ca\(^{2+}\) are known to result in the loss of mitochondrial function (10). By preventing the accelerated phospholipid depletion and associated microsomal and plasma membrane dysfunction, chlorpromazine prevents the influx into the cell and subsequent mitochondrial accumulation of Ca\(^{2+}\) that occurs with reperfusion. Normal mitochondrial structure and function can be restored when this Ca\(^{2+}\) accumulation is prevented.

Two mechanisms can account for most of the alterations of liver mitochondria with ischemia: the accumulation of long chain acyl-CoA esters or a de-energization of the inner membrane, or both. Long chain fatty acyl-CoA esters have been shown to be capable of reversibly inhibiting adenine nucleotide translocase when added \textit{in vitro} to liver or heart mitochondria (33–37). A positive correlation in liver has been reported between the tissue concentration of long chain acyl-CoA esters and inhibited translocation (38). Tissue levels of fatty acyl-CoA are normally low but increase during ischemia (38–40). Loss of adenine nucleotide translocase very likely results from the inhibitory effect of the accumulation of these long chain acyl-CoA esters. The presence of adenosine 3'-phosphate 5'-pyrophosphate in the acyl-CoA molecule may account for this inhibition (25).

Such an inhibition of adenine nucleotide translocase can account for the loss of respiratory control and DNP-activated ATPase in ischemic mitochondria and their recovery with reperfusion in the chlorpromazine pretreated animal. Our data document an apparent dependency in ischemic mitochondria of the respiratory control ratio on the activity of the adenine nucleotide translocator. It has been previously noted that the \(K_m\) for ADP for state 3 respiration in the presence of MgCl\(_2\) is of the same order of magnitude as the \(K_m\) value found for ATP (41). Addition of atractyloside to normal mitochondria \textit{in vitro} abolished respiratory control with ADP and inhibited mitochondrial ATPase. The inhibition of adenine nucleotide translocase produced \textit{in vitro} by fatty acyl-CoA esters is reversible upon addition of carnitine (28), which presumably converts these esters to fatty acyl carnitine. Repression of ischemic livers would produce a similar reduction in the concentration of fatty acyl-CoA esters by simply allowing resumption of their oxidation.

Inhibition of adenine nucleotide translocase by fatty acyl-CoA esters, however, cannot account for all of the effects of ischemia on liver mitochondria. Mitochondria treated with atractyloside respond normally with an increased \(O_2\) consumption upon addition of DNP. Ischemic mitochondria do not (Table VI). This loss of DNP-induced \(O_2\) consumption can be explained by a loss of the normal electrochemical gradient across the inner membrane with ischemia, a de-energization of the inner membrane. Electron transport and its associated \(H^+\) transport should cease in the absence of \(O_2\). Such a loss of the normal electrochemical gradient that must surely result from ischemia could relate to several of the functional alterations in ischemic mitochondria. It would explain the loss of DNP induced \(O_2\) consumption. It is possible that in part the loss of adenine nucleotide translocation and its associated loss of respiratory control may result from a loss of the mitochondrial membrane potential. Translocation of ADP into mitochondria with release of ATP into the cytoplasm requires movement of phosphate to maintain charge equilibrium. The \(H^+\) ion membrane potential supplies the energy for this phosphate movement. Uptake of phosphate diminishes the pH gradient, and agents such as uncouplers and ionophores which reduce or reverse this gradient also abolish the energy-dependent uptake of phosphate (42). An inability to translocate phosphate as a consequence of the loss of the inner membrane potential may limit, in turn, the ability to translocate adenine nucleotides. With reperfusion the mitochondria should be reoxygenated with a return of electron transport and regeneration of the inner membrane potential.

The ultrastructural alterations in Fig. 6, B and C may also be a manifestation of the de-energization of the mitochondria. It is well known that there are conformational changes in the inner membrane accompanying energization of the mitochondria as revealed by the use of fluorescent probes. Fig. 6, B and C, may illustrate the structural expression of these changes in inner membrane conformation. Such changes may also be the basis of the loss of the protein band revealed by polyacrylamide gel electrophoresis of ischemic mitochondrial proteins (Fig. 3). A change in membrane conformation accompanying de-energization could cause dissociation of these proteins from the membrane or allow their diffusion out of the inner matrix compartment.

We have no explanation at the present time for the loss of cytochromes aa\(_3\) and \(c + c_1\) (Fig. 2) nor of the nature of the protein(s) depleted from the acrylamide gels of ischemic mitochondria. The molecular weight of the depleted band makes it unlikely that it represents the missing cytochromes. In addition the recent report (43) that the adenine nucleotide translocator consists of \(M_1 = 33,000\) protein subunits makes it unlikely that the protein(s) \((M_1 = 83,000)\) is related to the adenine nucleotide translocator. That both the alterations in the cytochrome contents and in protein composition are direct consequences of the metabolic abnormalities associated with ischemia is suggested by their ready reversibility with reperfusion in the chlorpromazine-pretreated animals.

Lastly it is necessary to distinguish between the effects of ischemia on mitochondria \textit{in vivo} and the \textit{in vitro} process of mitochondrial “aging.” The storage of mitochondria at 4 to 30°C is commonly referred to as aging and is accompanied by a gradual loss of energy-linked functions that bear an obvious relationship to those occurring \textit{in vivo} with ischemia. Most of the recent studies of mitochondrial aging (44–50), however, suggest that fatty acids released from mitochondria and other particular contaminants present (such as microsomes) are responsible for the decline in respiratory control and ADP:O ratios under these conditions. In contrast to ischemic mitochondria, the decreases in the RCR and ADP:O ratios are primarily due to an increase in the state 4 respiration rate (45, 47, 49). Unsaturated long chain fatty acids have been shown to be potent uncoupling agents with a mode of action similar to that exhibited by DNP (51, 52). Free fatty acid levels.
increase with time after isolation of mitochondria (52). Free fatty acids added to normal mitochondria in concentrations similar to those which accumulate in mitochondria during in vitro aging significantly lower the RCR primarily by increasing state 4 respiration (53). All these studies suggest that endogenous phospholipase A₂ activity may be involved in the deterioration of mitochondria in vitro. Waite et al. (44) have shown a direct relationship between endogenous phospholipase A₂ activity and mitochondrial deterioration in vitro. Scarpia and Lindsay (45) demonstrated a protection against the effects of aging by the local anesthetic and powerful phospholipase inhibitor nupercaine.

Ischemia clearly produces a different pattern of mitochondrial alterations than those associated with in vitro aging. At least initially, aging more closely resembles that functional alteration produced by DNP, while ischemic mitochondria show an increase in state 4 respiration and are insensitive to this uncoupler. An elevated state 4 respiratory rate implies that aged mitochondria are, at least initially, still energized. Recently Parce et al. (50) have shown a persistence of energy-linked 1-anilino-8-naphthalenesulfonic acid fluorescence at a time when respiratory control was lost in mitochondria aged at 18°C.

Acknowledgments—We gratefully acknowledge the assistance of Dr. Colleen Smith throughout the course of this study and that of Ruth Shivers in the preparation of the manuscript.

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