The Mechanism of Stimulation of Respiration by Fatty Acids in Isolated Hepatocytes*

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Addition of fatty acids to isolated hepatocytes raised respiration rate by 92% and raised mitochondrial membrane potential (ΔΨm) in situ from 155 to 162 mV suggesting that the increased fuel supply had a greater effect on respiration rate than any increases in processes that consumed mitochondrial proton motive force (Δp). The relationship between ΔΨm and respiration rate was changed by addition of fatty acids or lactate, showing that there was also stimulation of Δp-consuming reactions. In the presence of oligomycin the relationship between ΔΨm and respiration rate was unaffected by substrate addition, showing that the kinetics of Δp consumption by the H⁺ leak across the mitochondrial inner membrane were unchanged. The stimulation of Δp consumers by fatty acids therefore must be in the pathways of ATP synthesis and turnover. Inhibition of several candidate ATP-consuming reactions had little effect on basal or fatty acid-stimulated respiration, and the nature of the ATP turnover reactions in hepatocytes remains speculative. We conclude that fatty acids (and other substrates) stimulate respiration in hepatocytes in two distinct ways. They provide substrate for the electron transport chain, raising Δp and increasing the non-ohmic proton leak across the mitochondrial inner membrane and the rate of oxygen consumption. They also directly stimulate an unidentified Δp-consuming reaction in the cytoplasm. They do not work by uncoupling or by stimulation of intramitochondrial ATP-turnover reactions.

The addition of substrates such as lactate, pyruvate, and fatty acids to perfused liver or isolated hepatocytes results in increased oxygen uptake. However, the extra respiration is greater than that required to satisfy the extra ATP demand for biosynthetic processes such as gluconeogenesis, protein synthesis, ureogenesis, and lipogenesis (1-9). This apparent loss in efficiency of oxidative phosphorylation is particularly evident when cells are given fatty acids. The effect occurs at physiological concentrations of fatty acids and results in heat production (10). It has been studied because of its potential importance in thermogenesis and the energy economy of the cell. Several mechanisms for this effect have been proposed but no consensus has been reached (11).

Fatty acids are known to uncouple respiration of mitochondria in quite low concentrations (12, 13), and uncoupling was suggested as the mechanism in perfused liver since fatty acids lowered the cytosolic ATP/ADP ratio and mitochondrial ΔpH (6, 8). However, the apparent uncoupling effect was not seen in the presence of albumin (which binds fatty acids so reducing their free concentration) although the respiration rate was still increased (6, 11). The most convincing evidence against uncoupling comes from the observation that inhibitors of ATP synthesis (such as oligomycin) greatly decrease the fatty acid stimulation of respiration (4, 14) suggesting that increased ATP turnover may be an important contributor to the increase in oxygen utilization. Rottenberg and Hashimoto (15) have proposed a modification of this uncoupling theory. They suggest that fatty acids are decouplers as they stimulate electron transport but do not decrease the mitochondrial membrane potential.

Plomp et al. (16) found that 50-60% of the extra oxygen consumption induced by addition of fatty acids to hepatocytes was prevented by ouabain and proposed that it was due to a direct stimulation of the (Na⁺ + K⁺)-ATPase by fatty acids. However, under the conditions used, respiration is inhibited by secondary effects of Na⁺ and K⁺ distribution, and the direct contribution to respiration of ATP turnover by the (Na⁺ + K⁺)-ATPase is very small (17).

Intramitochondrial ATP turnover by futile cycling during octanoyl-coenzyme A synthesis and degradation has been proposed to account for the stimulated oxygen uptake on addition of medium chain fatty acids to cells (18). However, this mechanism should be sensitive to oligomycin but not to carboxyatractyloside, an inhibitor of the adenine nucleotide translocase, contrary to what is observed (14). Other mechanisms that have been put forward to account for the fatty acid stimulation of respiration include reversed electron transport (5), energy requirement of metabolic and reducing equivalent translocation (1), and an increased rate of protein synthesis (2).

In this paper we document the effects of fatty acids on Δp in hepatocytes to distinguish between stimulation of electron transport by addition of substrate (which will raise Δp) and stimulation of electron transport by ATP turnover or uncoupling (which will lower Δp). We show that part of the extra oxygen consumption is due to increased substrate supply.

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1 The abbreviations used are: Δp, difference in electrochemical potential of protons across the mitochondrial inner membrane (proton motive force ΔΨm = ΔpH + Δψm); Δψm, electrical potential across the mitochondrial inner membrane (Ψm = −ψm); Δψp, potential across the plasma membrane (Ψp − ψp); ΔpH, pH difference across the mitochondrial inner membrane (pHΔ = pHl); BSA, bovine serum albumin; DMO, 5,5-dimethyl-2,4-dione. Subscripts "e," "c," "m," and "tot" refer to extracellular, cytoplasmic = nuclear, mitochondrial, and total intracellular pools, respectively.
which increases Δp and cycling of protons through the non-
ohmic proton leak across the mitochondrial inner membrane, and
part is due to cytoplasmic ATP turnover by unidentified
reactions. Uncoupling of respiration by fatty acids does not

MATERIALS AND METHODS

Preparation and incubation of hepatocytes, measurement of cell
volume, 1H]tyrosine/methylphosphonate; accumulation, 3Cl) and
14C]DMO distributions, measurement of plasma and mitochondrial
membrane potentials, and measurement of hepatocyte respiration
rates were carried out as in Refs. 19, 20. Hepatocytes were prepared
from female Wistar rats fed or 24-h fasted (to deplete liver glycogen).
Hepatocytes prepared from fed rats were incubated in medium
supplemented with 10 mM glucose.

Fatty acids (octanoate or palmitate) were neutralized with 0.1 M
NaOH and dissolved in 9% (w/v) defatted BSA which was previously
dialyzed against 153 mM NaCl, 10.8 mM KCl. BSA was defatted by
the method of Chen (21). Hepatocytes were incubated with final
concentrations of 2.25% (w/v) BSA and 2 mM palmitate, 3 mM
octanoate, or 10 mM lactate. 14 mg of 3-mercaptopticolineic acid was
dissolved in 1 ml of basic salts medium containing 25 mM NaHCO3.
The solution was neutralized by adding small aliquots of 10 M NaOH
giving a final stock solution of 100 mM.

L-[Lactate, palmitic acid, ruthenium red, cycloheximide, acti-
nomycin D, emetine, salicylhydroxamic acid, quercetin, lanthanum
chloride, and lithium chloride were from Sigma. o-Amanitin was from
BDH. Octanoic acid was from Hopkin and Williams. 3-Mercaptopi-
colinic acid was from Smith Kline and French. Other materials were
from the sources indicated in Ref. 19. Water-insoluble compounds
were dissolved in dimethyl sulfoxide. Statistical significances were
determined by Student’s t test.

RESULTS AND DISCUSSION

By conceptually dividing the respiratory system into those
reactions that produce Δp and those that consume Δp, we are
able to analyze whether substrate addition stimulated respi-
ration by increasing the Δp-producing pathways (which would
raise Δp) or by increasing the Δp-consuming pathways (which
would lower Δp). In these experiments we used hepatocytes
from fasted rats as the metabolic processes occurring are
simple: fatty acids are oxidised to ketone bodies and there is
little lipid synthesis or formation of triacylglycerols (1). As
substrate we used palmitate, octanoate, or lactate; all three
result in oxygen uptake in excess of the requirement for
 gluconeogenesis (1). To simplify the analysis using lactate, 3-
mercaptopicolinate was added to prevent gluconeogenesis, as
does in perfused livers (22, 23); Fig. 1 confirms that the
 inhibitor was also effective in isolated hepatocytes; 1 mM 3-
mercaptopicolinate abolished glucose production from lactate.

Stimulation of Δp Producers by Fatty Acid Addition—Table
II shows that addition of octanoate stimulated cell respiration
by 92%. The major component of Δp in hepatocytes is Δψm
(19). Table I shows that the increased respiration rate was
associated with an increase in Δψm, showing that octa-
noate addition stimulates production of Δψm (and presumably
of Δp) more than it stimulates consumption of Δψm. This
effect of octanoate probably is due to an increased supply of
substrate for β-oxidation, resulting in increased NADH and
FADH supply and increased entry of electrons into the respira-
tory chain. A raised NADH/NAD ratio following fatty
acid addition has been demonstrated (5), and we have shown
that an artificially raised NADH/NAD ratio is sufficient to
stimulate oxygen consumption by hepatocytes in the absence
of other effects. We have also shown (19) that raised Δψm
drives increased H+ backflow across the mitochondrial inner

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TABLE I

Comparison of respiration rate, \( \Delta \psi_m \), \( \Delta \psi_p \), \( f^{[4]} \)DMO distribution, and cell volume of hepatocytes respiring on different substrates in the presence and absence of antimycin.

Hepatocytes from fasted rats (15-20 mg wet weight/ml) were incubated for 40 min in 2 ml of medium containing 108.5 mM NaCl, 5.2 mM KCl, 25 mM NaHCO\(_3\), 0.41 mM MgSO\(_4\), 10.42 mM NaHPO\(_4\), 2.5 mM CaCl\(_2\), 0.001% (w/v) phenol red, and 2.25% (w/v) defatted BSA. The gas-phase above the cells was 95% air and 5% CO\(_2\) to allow equilibration of the medium to a pH of 7.4. Hepatocytes incubated with 10 mM lactate had 1 mM 3-mercaptopicolinate present to prevent glucose production. \( + \) antimycin refers to a concentration of 0.25 \( \mu \)M for cells incubated with 10 mM lactate and 0.1 \( \mu \)M for cells incubated with 3 mM octanoate. \( * \) significantly different (\( p < 0.01 \)) from the value in the absence of antimycin. \( \dagger \) significantly different from value in the absence of added substrate. (Other comparisons i.e. \( \pm \) antimycin for each substrate and endogenous substrate versus octanoate and endogenous versus lactate) were not significantly different (\( p > 0.2 \)). The data are means \( \pm \) S.E. with number of experiments in parentheses. For each antimycin concentration \( \Delta \psi_m \) was calculated using the corresponding value of \( \Delta \psi_p \) in the absence of antimycin.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Endogenous</th>
<th>Octanoate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu )mol O(_2)/min/g wet wt</td>
<td>( \delta \psi_m ) (mV)</td>
<td>( \delta \psi_m ) (mV)</td>
<td>( \delta \psi_m ) (mV)</td>
</tr>
<tr>
<td>No antimycin</td>
<td>1.34 ( \pm ) 0.05 (5)</td>
<td>2.57 ( \pm ) 0.07 (5) ( \dagger )</td>
<td>0.76 ( \pm ) 0.02 (3)</td>
</tr>
<tr>
<td>+ antimycin</td>
<td>1.44 ( \pm ) 0.09 (5) ( \dagger )</td>
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</tbody>
</table>

Fig. 2. Relationship between \( \Delta \psi_m \) and respiration rate for cells incubated with endogenous substrate, octanoate, or lactate. Hepatocytes were prepared as described in Table I in the presence of different concentrations of antimycin (0, 0.10, 0.15, 0.25, and 0.25 \( \mu \)M for cells respiring on endogenous substrates, 0, 0.16, 0.2, and 0.25 \( \mu \)M for cells respiring on 10 mM lactate, and 0, 0.05, 0.075, and 0.10 \( \mu \)M for cells incubated with 3 mM octanoate). 1 mM 3-mercaptopicolinate was included in incubations containing lactate. Data are means \( \pm \) S.E. for duplicate determinations of \( \Delta \psi_m \) and a single determination of respiration rate for three or four separate hepatocyte preparations. Simultaneous measurement of cell volume was made for each antimycin concentration.

\(< 0.05 \). This result shows that octanoate stimulates respiration by a direct effect on \( \Delta \psi \)-producing reactions and so does lactate in the presence of 3-mercaptopicolinate.

Table I reports control experiments to test whether the shifted relationship between \( \Delta \psi_m \) and respiration rate with octanoate might be caused by changes in \( \Delta \psi_p \) or in \( \Delta \psi_m \). \( \Delta \psi \), will affect triphenylmethylphosphonium\(^*\) distribution and hence the measurement of \( \Delta \psi_m \); however, \( \Delta \psi_m \) was not altered by octanoate either in the absence of antimycin or in the presence of the highest antimycin concentration used in Fig. 2. Changes in \( \Delta \psi_m \) might have occurred, so that the contribution of \( \Delta \psi_m \) to \( \Delta \psi \) might have altered with octanoate, thus causing the shift in the curve in Fig. 2. We tested whether this was so by measuring the distribution of DMO assuming no changes in cytosolic pH changes in DMO distribution will reflect changes in \( \Delta \psi_m \) (see Ref. 19). Table I shows that DMO distribution was unchanged after addition of octanoate with or without antimycin. The results of similar controls using lactate were slightly different; lactate increased both \( \Delta \psi_m \) and \( \Delta \psi_p \). However, since a 1 mV error in \( \Delta \psi_p \) causes a 1 mV error in \( \Delta \psi_m \), these changes were not great enough to have caused the displacement of the curve for lactate in Fig. 2.

Having established that fatty acids stimulate not only \( \Delta \psi \)-producing reactions but also \( \Delta \psi \)-consuming reactions, we investigated which \( \Delta \psi \)-consuming reactions were affected. These may be divided into those causing \( H^+ \) flow across the mitochondrial inner membrane during ATP formation (ATP synthase and all subsequent steps) and those causing \( I^+ \) flow across the membrane by other pathways (primarily the \( H^+ \) leak, but also including \( I^+ \)-linked cycling processes, for example, cation cycling).

Effects of Fatty Acids on \( H^+ \) Leak—To study the effects on the kinetics of the \( H^+ \) leak, we prevented \( H^+ \) flux through ATP synthase using oligomycin and analyzed whether the relationship between \( \Delta \psi_m \) and respiration rate (i.e. the kinetics of the \( H^+ \) leak) was changed by addition of fatty acids (see Refs. 19, 25). Hepatocytes from fasted rats rapidly lose viability if the ATP synthase is inhibited (19); therefore, these experiments were carried out on cells from fed animals which are able to maintain their ATP levels by glycolysis (19).
FIG. 3. Relationship between $\Delta\varphi_m$ and respiration rate in the presence of oligomycin, for cells incubated with 10 mM glucose, glucose plus 3 mM octanoate, or glucose plus 2 mM palmitate. Hepatocytes were prepared from fed rats and incubated as described in Fig. 3. $\Delta\varphi_m$ was determined from the distribution of DMO. Results are means ± S.E. of duplicate determinations from three separate hepatocyte preparations.

shows that this non-ohmic relationship is superimposable in the presence and absence of fatty acids but extended to a faster respiration rate and higher membrane potential in the presence of fatty acids. Thus, there is an increase in the rate of $H^+$ leak across the inner mitochondrial membrane due to a slightly raised $\Delta\varphi_m$ in the presence of fatty acids, as predicted above, but no evidence for direct uncoupling by fatty acids. The direct stimulation of $\Delta p$ consumers identified in Fig. 2 must therefore be in the ATP synthase and ATP-consuming pathways. ($\Delta$ stimulation of the ATP synthase might include a decoupling-type mechanism as suggested by Rottenberg and Hashimoto (15)).

Control experiments were performed in which $\Delta\varphi_p$ was measured using the distribution of $^{36}$Cl$^-$ under all the conditions presented in Fig. 3. $\Delta\varphi_p$ shows a relationship between plasma membrane potential and respiration rate for cells titrated with myxothiazol in the presence of oligomycin and incubated with 10 mM glucose alone, with glucose plus 2 mM palmitate or with glucose plus 3 mM octanoate. For each substrate there was no change in $\Delta\varphi_p$ as respiration was inhibited. We observed lowered $\Delta\varphi_p$ values for cells incubated with palmitate. It has been shown previously that the fasting state results in a depolarization of $\Delta\varphi_p$ by about 7 mV (27). The reason for this depolarization is unknown. Fig. 5 shows that there is little change in DMO distribution under the same conditions. If we assume a constant cytoplasmic pH of 6.9 (28), the slight fall in DMO distribution in the presence of octanoate would correspond to only a 10 mV decrease in $\Delta\varphi_m$ (calculated as in (19)), which would not affect the conclusions from Fig. 3. Alternatively $\Delta\varphi_m$ might remain unchanged in the presence of octanoate, and the measured difference in DMO distribution would be due to an acidification of the cytoplasm of 0.08 units, again with no change in the conclusions from Fig. 3.

Nature of Fatty Acid-stimulated ATP Turnover—We have not established which steps within the ATP turnover reactions are directly stimulated by addition of fatty acids or lactate. Direct effects on the ATP synthase or the adenine nucleotide translocase are possible. We can exclude stimulation of intramitochondrial ATP consumption as proposed by Schonfeld et al. (18) since inhibition of the adenine nucleotide translocase by 200 $\mu$M carboxyatractyloside gave respiration rates for cells from fed rats in the presence of either octanoate or palmitate that were not significantly different ($p > 0.1$) from the respiration rates in the presence of oligomycin, confirming our earlier results from fasted animals (14). Direct activation of intramitochondrial ATP turnover by fatty acids would have given increased respiration rates that were insensitive to carboxyatractyloside but sensitive to oligomycin. We then investigated which cytoplasmic ATP-consuming processes might contribute to fatty acid stimulation of respiration using cells prepared from fed rats. Hepatocytes were incubated with a number of inhibitors of ATP-consuming reactions as described in Table II. The results clearly indicate that fatty acid stimulation of respiration cannot be accounted for by increased rates of protein synthesis, RNA synthesis, ATP-dependent proteolysis, quercetin-sensitive ATPase, Ca$^{2+}$-ATPase, inositol phosphate turnover or (Na$^+$ + K$^+$)-ATPase (see also Ref. 17). Thus, the nature of the ATP-consuming reactions in hepatocytes and the reactions stimulated on fatty acid addition remains unresolved. This observation and conclusion are similar to our results with thyrocytes (33) but differ from the results obtained in reticulocytes and in Erhlich ascites tumor cells, where a large proportion of the ATP consumption by the cells has been accounted for (29, 30).

Recent work by Baranyai and Blum (34) has shown that futile cycling in the glucose/glucose 6-phosphate, glycogen/
glucose 6-phosphate, fructose 6-phosphate/fructose 1,6-bisphosphate and phosphoenolpyruvate/pyruvate/oxaloacetate substrate cycles may account for 22% of cellular ATP production in hepatocytes from fed rats incubated with a number of substrates (not including fatty acids). Energy wasting futile cycles were proposed to account for the low apparent ADP/O ratios observed during fatty acid oxidation in perfused livers by Debeer et al. (4). In the absence of an identified ATP-consuming process to account for the fatty acid stimulation of respiration, futile cycling at these loci remains a possible major contributor to both the basal oxygen consumption and the extra oxygen consumption caused by substrate addition.

Conclusions—The results presented in this paper show that fatty acids stimulate the respiration of hepatocytes by two distinct mechanisms. 1) They stimulate production of \( \Delta p \) by acting as substrates, increasing NADH/NAD ratios (5) and raising \( \Delta p \). This increase in \( \Delta p \) results in an increase in the non-ohmic H\(^+\) leak first identified in intact cells in Ref. 19, and an increase in respiration rate. 2) They also stimulate \( \Delta p \) consumers at an unidentified step or steps within the pathways of ATP turnover. If stimulation is of ATP consumption directly, then it is cytoplasmic and not intramitochondrial since it is prevented equally well by inhibition of the adenine nucleotide translocase or the ATP synthase. We find no evidence for any direct uncoupling effect of fatty acids under our conditions (Fig. 3). Lactate also appears to stimulate respiration by directly affecting ATP turnover in the presence of 3-mercaptopicolinate to prevent gluconeogenesis. This case it is possible that lactate causes ATP consumption during the conversion of lactate to malate through pyruvate carboxylase, as 3-mercaptopicolinate may inhibit gluconeogenesis at phosphoenolpyruvate carboxykinase (22, 23) leaving pyruvate directly, then it is cytoplasmic and not intramitochondrial.

We can estimate the proportion of the extra oxygen consumption following addition of fatty acids that is used to drive the increased proton leak as described (19). The respiration rates in the absence of oligomycin for cells respiring on glucose, octanoate, and palmitate are 1.71 ± 0.08 (7), 3.05 ± 0.3 (4), and 2.81 ± 0.22 (3), respectively (means ± S.E.). From the corresponding respiration rates in the presence of oligomycin (Fig. 3), we calculate that 24 and 29% of the extra oxygen consumption on addition of octanoate and palmitate, respectively, is due to increased H\(^+\) leak (assuming that there is no change in \( \Delta W \) or \( \Delta P_{\text{H+}} \) on addition of oligomycin). Similarly about 29% of the total oxygen consumption under all three conditions is used to drive the H\(^+\) leak. This data is obtained from cells of fed rats. We cannot extrapolate these results to explain or quantify the stimulation of respiration by fatty acids in hepatocytes from fasted rats. However, we do observe a higher value of \( \Delta W \) on addition of octanoate to hepatocytes from fasted rats and so propose an increased proton leak in these cells also.

There have been speculations about the physiological role of the increased oxygen consumption of liver when presented with fatty acids (5, 7). One possibility that has not been discussed is that increased H\(^+\) flux into the mitochondrial matrix, due to either increased \( \Delta p \) or to increased ATP turnover, would allow electron transport and \( \beta \)-oxidation to proceed at high rates independently of ATP demand. This could be important in allowing continued production of ketone bodies by the liver in starvation.

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References