THE INHIBITION OF ADENINE NUCLEOTIDE TRANSLOCASE ACTIVITY BY OLEOYL CoA AND ITS REVERSAL IN RAT LIVER MITOCHONDRIA

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SUMMARY

The inhibitory effect of oleic acid on the $^{32}P_i$-ATP exchange activity and ^{14}C-ADP translocation through the mitochondrial membrane can be reversed by substrates preferentially utilizing CoA and thereby preventing acylation of the fatty acid. By contrast the inhibition produced by oleoyl CoA can be reversed only when carnitine is added to augment oxidation of the CoA ester. The slow rate of respiration and sluggish response to ADP in oleoyl CoA treated mitochondria is overcome by the uncoupler, salicylanilide XIII. Inhibition of translocation of adenine nucleotides through the inner mitochondrial membrane by oleoyl CoA could regulate metabolism by inducing a transition from state 3 to state 4 respiration.

INTRODUCTION

In a recent report from this laboratory we postulated that the sluggish rate of oxygen consumption by liver mitochondria of the hibernating ground squirrel was due to a decrease in the penetration of ADP (1). Wojtczak and Zaluska previously observed an inhibition of translocation of adenine nucleotides through rat liver mitochondrial membranes by oleic acid (2). The fasting hibernator is primarily dependent upon fatty acid oxidation for energy requirements and increased levels of lipid would be expected to accumulate in the liver. In the present communication an *in vitro* system was developed using rat liver mitochondria to simulate the conditions occurring during hibernation. Inhibition of $^{32}P_i$-ADP exchange activity and translocation of ADP was found

to be due to long chain fatty acyl CoA esters rather than the fatty acid itself. The site of inhibition was localized to the inner mitochondrial membrane. In addition stimulation by uncouplers of the initial low respiratory rate in the hibernating squirrel could be reproduced in isolated rat liver mitochondria previously incubated with oleoyl CoA.

MATERIALS AND METHODS

Rat liver mitochondria prepared by the method of Schneider (3) were suspended in 0.25 M sucrose at a concentration equivalent to 1.0 gm liver per ml. Oxygen consumption was determined on a Gilson oxygraph using a Clark electrode. Respiratory control ratios were calculated according to Estabrook (4). $^{32}P_i$-ATP exchange activity was determined by the method of Boyer et al.(5) and translocation of adenine nucleotides according to Wojtczak and Zaluska (2). Acetoacetate was prepared by standard published procedures (6). Protein was determined by the biuret method (7). $^{32}P_i$ was purchased from New England Nuclear, ^{14}C-ADP from Schwartz-Mann, acyl CoA derivatives from P.L. Biochemicals and atractyloside from Calbiochem. Salicylanilide XII was a gift from Dr. H.A. Lardy. All other reagents were of the highest grade commercially available.

RESULTS AND DISCUSSION

Falcone and Mao (8) demonstrated that long chain fatty acids at concentrations too low to produce uncoupling of respiratory chain oxidative phosphorylation and stimulation of ATPase activity could, in the presence of KCN, inhibit the $^{32}P_i$-ATP exchange activity of mitochondria. In the present experiments oleic acid at a concentration of 0.03 mM, considerably less than necessary for formation of micelles(9), inhibits the $^{32}P_i$-ATP exchange reaction by 70% when KCN is added to prevent oxidation of the

TABLE I

Inhibition of $^{32}P_i$-ATP exchange activity by oleic acid and oleoyl CoA, and reversal of inhibition by α ketoglutarate and carnitine. The basic incubation mixture in 1.0 ml contained 10 mM ATP, 10 mM P_i, 75 mM tris-HCl, pH 7.0, and 45 mM sucrose. Additions were 1.0 mM KCN, 0.03 mM oleic acid, 0.03 mM oleoyl CoA, 5 mM α ketoglutarate and 5 mM D,L-carnitine. Total radioactivity in P_i was equal to 20,000 c.p.m. The reaction was initiated with 2.5 mg mitochondrial protein and incubated at 20° for 15 minutes.

ADDITIONS	$^{32}P_i$-ATP EXCHANGE ACTIVITY cpm/μmole ATP
EXPERIMENT 1	
NONE	452
KCN	341
OLEIC ACID	406
OLEIC ACID + KCN	93
OLEIC ACID + KCN + αKETOGLUTARATE	424
OLEIC ACID + KCN + ACETOACETATE	98
EXPERIMENT 2	
NONE	401
OLEOYL CoA	51
CARNITINE	420
OLEOYL CoA + Carnitine	376
OLEOYL CoA + α KETOGLUTARATE	60

fatty acid (Table 1). Besides bovine serum albumin, certain keto acids such as α ketoglutarate, which had no effect on basal exchange, could reverse the inhibition of oleic acid. Pyruvate and oxalacetate produced identical results while other Krebs cycle intermediates were ineffective. The most likely explanation is that these compounds even in the presence of cyanide could act as substrates for the pyruvate or α ketoglutarate dehydrogenase reactions which would preferentially utilize endogenous CoA and prevent acylation of the fatty acid. The lack of effect of acetoacetate rules out a block in the electron transport chain which might be overcome by oxidation of NADH through β hydroxybutyrate, malate or glutamate dehydrogenase. In the second part of this experiment it is conclusively shown that oleoyl CoA rather than oleic acid accounts for the inhibition. Oleoyl CoA even without

cyanide causes a 90% inhibition of exchange activity which can be almost completely reversed with carnitine. Addition of α keto acids are ineffective indicating that their protective effect is prior to the formation of the CoA ester.

In Table II the translocation of ADP across the mitochondrial membrane was tested directly under conditions similar to those used in the $^{32}P_i$-ATP exchange experiment. Neither KCN nor oleic acid alone inhibited. Presumably at this low concentration the fatty acid was completely metabolized and never reached an inhibitory level. However, on addition of cyanide, there was a 50% inhibition of ADP transport which could be overcome with α ketoglutarate. In the second part of this experiment, which was run

TABLE II

Inhibition of ^{14}C-ADP translocation through the mitochondrial membrane by oleic acid and oleoyl CoA, and the reversal of inhibition by α ketoglutarate and carnitine. The basic reaction mixture contained 100 mM KCl, 40 mM tris-HCl, pH 7.4, 1.0 mM $MgCl_2$. Additions were 0.3 mM KCN, 0.03 mM oleic acid, 0.03 mM oleoyl CoA, 5 mM α ketoglutarate, 5 mM D,L-carnitine, and 0.003 mM atractyloside. The reaction mixture was pre-incubated with 2.5 mg mitochondrial protein for 4 minutes at the temperatures indicated. The reaction was then initiated by the addition of 0.08 mM ^{14}C-ADP (40,000 cpm). After 2 minutes, the translocation of adenine nucleotides was terminated with atractyloside.

ADDITIONS	RADIOACTIVITY IN MITOCHONDRIA cpm
EXPERIMENT 1 25°	
NONE	28,800
KCN	26,705
OLEIC ACID	28,510
α KETOGLUTARATE	28,565
OLEIC ACID + KCN	13,695
OLEIC ACID + KCN + α KETOGLUTARATE	29,796
EXPERIMENT 2 0°	
NONE	14,300
OLEIC ACID	14,285
OLEOYL CoA	1,650
CARNITINE	14,000
OLEOYL CoA + CARNITINE	11,850
ATRACTYLOSIDE	1,754

at 0° to prevent complete oxidation of the oleoyl CoA, marked inhibition of the translocase was observed. Reversal of the inhibition by carnitine indicates the site of inhibition at the inner mitochondrial membrane. Transacylation of the fatty acyl CoA by acyl carnitine transferase permits transport as the carnitine ester and complete oxidation of the reformed acyl CoA (10). The known effect of atractyloside on adenine nucleotide transport is shown for comparison (11).

In previous studies it was shown that the low rate of oxygen consumption by mitochondria of the hibernating ground squirrel could be stimulated to a much greater extent by an uncoupler than by ADP (1). In the present experiments oleoyl CoA inhibition of the ADP stimulation of oxygen uptake was overcome by the potent uncoupler, salicylanilide XIII (Figure 1). The poor penetration of ADP is bypassed by the uncoupling of endogenous phosphorylation from respiration. With a somewhat different approach, similar conclusions have been reached by Pande and Blanchaer (12). In

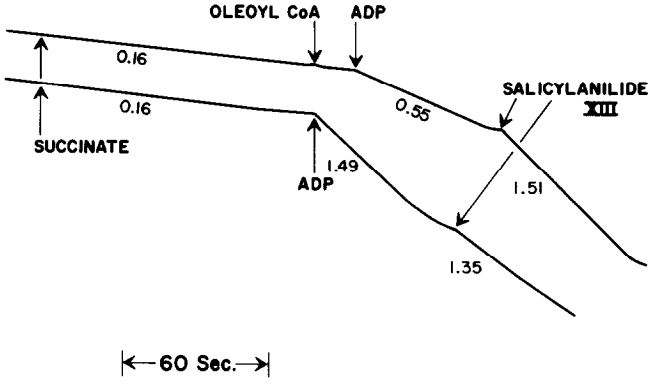

FIGURE 1 Control of respiration and response to oleoyl CoA by rat liver mitochondria. Liver mitochondria (7.0 mg protein) was added to 2.0 ml reaction mixture containing 20 mM KCl, 225 mM sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$ and 20 mM triethanolamine-HCl, pH 7.4. At the points indicated, 2.0 mM succinate was added followed by additions of 0.03 mM oleoyl CoA, 0.35 mM ADP, and 0.2 μM salicylanilide XIII. The numbers in the O_2 tracing represent the respiration rate expressed in micromoles of oxygen per mg protein per hour.

this communication only the experiments with oleic acid and oleoyl CoA are presented. However, in studies to be reported in detail, identical results were obtained with the fatty acids and CoA esters corresponding to those known to effect $^{32}P_i$-ATP exchange and ATPase activity (8).

These studies show that long chain acyl CoA esters can play a central role in the control of energy-linked mitochondrial respiration by reversibly blocking adenine nucleotide translocase activity. Added importance may be placed upon these observations because the nucleotide specificity of oxidative phosphorylation appears to reside at the level of the adenine nucleotide translocator (13). Inhibition of ADP translocation induces a transition from state 3 to state 4 respiration and an increased level of reduced pyridine nucleotides (14). One proposed mechanism for the stimulation of gluconeogenesis by fatty acid oxidation is the generation of necessary reducing equivalents which might be brought about by a state 4-like reduction of NAD. While in vitro results in themselves are inconclusive, findings such as those reported in the hibernator (1) lend credence to this hypothesis. Further studies using the diabetic rat liver as the pathophysiological model are now in progress.

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