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Effect of long-chain fatty acyl-CoA on mitochondrial and cytosolic ATP/ADP ratios in the intact liver cell

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The effect of long-chain acyl-CoA on subcellular adenine nucleotide systems was studied in the intact liver cell. Long-chain acyl-CoA content was varied by varying the nutritional state (fed and starved states) or by addition of oleate. Starvation led to an increase in the mitochondrial and a decrease in the cytosolic ATP/ADP ratio in liver both *in vivo* and in the isolated perfused organ as compared with the fed state. The changes were reversed on re-feeding glucose in liver *in vivo* or on infusion of substrates (glucose, glycerol) in the perfused liver, respectively. Similar changes in mitochondrial and cytosolic ATP/ADP ratios occurred on addition of oleate, but, importantly, not with a short-chain fatty acid such as octanoate. It is concluded that long-chain acyl-CoA exerts an inhibitory effect on mitochondrial adenine nucleotide translocation in the intact cell, as was previously postulated in the literature from data obtained with isolated mitochondria. The physiological relevance with respect to pyruvate metabolism, i.e. regulation of pyruvate carboxylase and pyruvate dehydrogenase by the mitochondrial ATP/ADP ratio, is discussed.

The CoA derivatives of long-chain fatty acids are known to inhibit adenine nucleotide transport in isolated mitochondria (Pande & Blanchaer, 1971; Shug *et al.*, 1971; for reviews see Vignais, 1976; Wojtczak, 1976) or liposomes (Woldegrigoris *et al.*, 1981). The inhibitory action has been shown to be specific for long-chain acyl-CoA, since the effective concentration for inhibition by non-esterified fatty acids or detergents is 1–2 orders of magnitude higher (Vignais, 1976; Wojtczak, 1976).

As pointed out by Wojtczak (1976) 'in order to prove or disprove conclusively the assumption that long-chain acyl-CoA inhibit adenine nucleotide translocation *in situ*, it should be necessary to show whether or not an increase in cellular long-chain acyl-CoA produces a decrease of the cytoplasmic and an increase in the mitochondrial phosphorylation potential . . .'. This would be similar to effects of classical inhibitors such as carboxyatractyloside (Zuurendonk & Tager, 1974; Soboll *et al.*, 1978). Attempts to calculate mitochondrial and cytosolic phosphorylation potentials by means of the metabolite indicator method have been made in rat livers from fed and starved animals (McLean *et al.*, 1971; Seitz *et al.*, 1977). Direct measurements of mitochondrial and cytosolic adenine nucleotide contents in hepatocytes from fed rats incubated with oleate showed an increase in the mitochondrial ATP/ADP ratio (Siess & Wieland, 1975). Moreover, in perfused livers from starved rats, we found increased mitochondrial and decreased cytosolic ATP/ADP ratios compared with livers from fed rats (Soboll *et al.*, 1978). Since the tissue contents of long-chain acyl-CoA are elevated after the addition of oleate and in livers from starved animals, the previous findings are consistent with an inhibition of mitochondrial adenine nucleotide transport by this metabolite. A conclusive proof, however, is still missing. This led us to study the effect of long-chain acyl-CoA on hepatic energy metabolism in more detail *in vivo*, in perfused livers and in isolated hepatocytes. Mitochondrial and cytosolic adenine nucleotide contents were determined by the fractionation technique in non-aqueous solvents as well as by the fractionation with digitonin (Elbers *et al.*, 1974; Zuurendonk & Tager, 1974). The results confirm the hypothesis that long-chain acyl-CoA exerts a

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specific and significant effect on the cellular energy state also in the intact cell, and therefore contributes to the regulation of cellular metabolism.

Methods

Extraction of the liver in vivo

Male rats weighing 200–250g were fed on stock diet (Altromin, Lage, Germany), or starved for 48h, or starved for 48h and re-fed *ad libitum* with pure glucose pellets for 1h. Rapid liver sampling was performed by the double-hatchet method (Faupel *et al.*, 1972). By this method liver samples were taken from unanaesthetized unrestrained rats and freeze-clamped within 3s.

Haemoglobin-free liver perfusion

Livers obtained from fed or starved animals were perfused with haemoglobin-free Krebs–Henseleit bicarbonate buffer in an open system (Krebs & Henseleit, 1932; Scholz *et al.*, 1973). Substrates were added as indicated in Tables and Figures. After 1h the perfusion experiment was terminated by freeze-clamping.

Fractionation of tissue in non-aqueous solvents

For determination of mitochondrial and cytosolic contents of ATP and ADP, the freeze-clamped livers were ground in liquid N_2 and freeze-dried at 0.26Pa at −40°C. About 0.3g of the freeze-dried powder was sonicated in a mixture of heptane and CCl_4 in 5s intervals and cooled continuously in heptane/solid CO_2 as described by Soboll *et al.* (1980), and then fractionated on density gradients consisting of heptane/CCl_4 mixtures (density range 1.28–1.38kg/l). The procedure was slightly modified for livers extracted *in vivo* (Schwenke *et al.*, 1981). The gradient yielded eight fractions each containing different proportions of mitochondrial and cytosolic protein. ATP and ADP contents and specific activities of marker enzymes (citrate synthase for mitochondria; phosphoglycerate kinase for cytosol) were measured enzymically (Kosicki & Srere, 1961; Bücher, 1955). On the basis of activities of marker enzymes and of adenine nucleotide contents in each fraction of the density gradient, mitochondrial and cytosolic ATP and ADP contents were calculated by extrapolation to mitochondria and cytosol respectively (Elbers *et al.*, 1974). They were converted into concentrations by assuming mitochondrial and cytosolic water contents of 0.8 and 3.8 μl/mg of compartmental protein respectively (Soboll *et al.*, 1976).

Isolation and incubation of hepatocytes

Hepatocytes were isolated from livers from fed rats as described by Berry & Friend (1969) with slight modifications (Sies *et al.*, 1977). For incubation hepatocytes were transferred into 25ml conical flasks containing incubation medium at 37°C and equilibrated with O_2/CO_2 (19:1). Final substrate concentrations were: glucose 10mM; L-lactate 2.1mM; pyruvate 0.3mM; DL-hydroxybutyrate 0.6mM; acetoacetate 0.3mM; stock solutions of oleate, 20mM in 2–20% (w/v) albumin (fraction V; fatty acid-free; Behring Werke), were added to give final fatty acid concentrations in the range 0.5–2mM; to the control incubation 0.2–2% (w/v) albumin was added. The incubation time was 25min (40min with carboxyatractyloside), shaking frequency 120min^{-1}.

Digitonin fractionation

The digitonin fractionation procedure (Zuurendonk & Tager, 1974) was used with the following modifications: 0.2ml of cells was added to 1ml of separation medium, mixed, and 0.7ml of the mixture was layered on top of the silicone oil in an Eppendorf cup containing 0.12ml of 1.88M-$HClO_4$ (bottom) and 0.5ml of the oil. After centrifugation, 0.5ml of the supernatant was deproteinized and neutralized. The fraction was designated 'cytosolic'. After removal of the remaining supernatant and the silicone oil, 0.12ml of $HClO_4$ was added to the pellet, mixed thoroughly, centrifuged and neutralized. The fraction was designated 'mitochondrial'. The composition of separation medium, the silicone oil and the extraction with $HClO_4$ were the same as described by Sies *et al.* (1977). In mitochondrial and cytosolic fractions ATP and ADP were measured (Soboll *et al.*, 1978).

Determination of oxygen uptake in isolated hepatocytes

Hepatocyte suspension (1ml) was transferred to a thermostatically controlled cylindrical plastic chamber connected with a Clark platinum oxygen electrode and a recorder. The chamber was closed and oxygen uptake recorded for 3min at 37°C while the suspension was stirred with a magnetic stirrer.

Determination of long-chain acyl-CoA

Long-chain acyl-CoA was determined in the acid-insoluble extract from liver tissue or hepatocyte suspension (Michael & Bergmeyer, 1974) after alkaline hydrolysis, by measurement of the CoA liberated, by using the kinetic assay method.

Materials

Enzymes and coenzymes were purchased from Boehringer (Mannheim, Germany). Sodium pentobarbital (Nembutal) was from Abbott (Neuilly-sur-Seine, France). Albumin was from Behring Werke (Marburg, Germany). All other chemicals

Table 1. *Effect of starvation and re-feeding on cytosolic and mitochondrial ATP/ADP ratios in rat liver in vivo*
Rats were starved for 48 h, or starved for 48 h and re-fed with pure glucose for 1 h; controls received a standard chow diet. Data are mean values ± S.E.M. for the numbers of experiments in parentheses. $*P<0.05$, $**P<0.01$, $***P<0.005$ for starved versus fed, or re-fed versus starved.

	Fed (7)	Starved 48 h (7)	Re-fed 1 h (5)
Total (μmol/g dry wt.)			
ATP	11.5±0.6	9.4±0.4	9.5±0.2
ADP	2.9±0.2	3.0±0.2	2.7±0.1
ATP/ADP	4.1±0.2	3.2±0.1***	3.6±0.1**
Mitochondrial (mM)			
ATP	6.0±0.8	7.5±0.7*	4.0±0.9**
ADP	7.6±1.2	7.5±0.9	7.2±0.2
ATP/ADP	0.85±0.1	1.0±0.04***	0.60±0.1*
Cytosolic (mM)			
ATP	5.9±0.4	4.9±0.2	5.0±0.2
ADP	0.82±0.1	0.87±0.1	0.73±0.06*
ATP/ADP	7.0±0.3	5.8±0.4*	7.0±0.4*

Table 2. *Mitochondrial and cytosolic ATP/ADP ratios in perfused livers from fed and starved rats*
Results are means ± S.E.M. for the numbers of experiments in parentheses. Substrates were infused for 20 min before the liver was freeze-clamped at 60 min of perfusion.

	ATP/ADP	
	Mitochondrial	Cytosolic
Fed, no substrates (9)	0.18±0.02	10.3±1.4
Starved, no substrates (6)	0.70±0.05	2.9±0.2
Starved, glucose 25 mM, glycerol 1 mM (4)	0.31±0.03	9.8±1.9
Fed: oleate 0.5 mM, albumin 2.0% (4)	0.72±0.07	8.5±1.2
Fed: octanoate 0.5 mM, albumin 2.0% (2)	0.18	10.9

were from Merck (Darmstadt, Germany) and were of the highest purity available.

Results

ATP/ADP ratios in the liver in vivo

Total and subcellular ATP and ADP contents in livers from rats fed on chow diet, starved for 48 h or starved for 48 h and re-fed with glucose for 1 h are shown in Table 1. The ATP content and the ATP/ADP ratios in the unfractionated tissue were lower in the starved than in the fed animals; the values for re-fed rats were between the values for fed and starved rats. In contrast with the overall values, the mitochondrial ATP concentration was highest in the starved state and lowest after carbohydrate re-feeding. The ADP concentrations were similar for the three metabolic conditions; consequently the mitochondrial ATP/ADP ratio was highest in the starved state and lowest after re-feeding. The cytosolic ratios resembled the values in the unfractionated tissue: the ATP concentration was decreased in livers from starved rats and after re-feeding; the ATP/ADP ratio was decreased in the starved state and identical in livers from fed and from re-fed rats. Long-chain acyl-CoA contents were 105±15, 390±80 and 155±25 nmol/g of protein in the fed, starved and re-fed states respectively.

ATP/ADP ratios in perfused livers

Long-chain acyl-CoA contents and subcellular ATP/ADP ratios were measured in isolated livers from fed and starved rats perfused without substrates and in livers from starved rats perfused with glucose and glycerol (Table 2). The three metabolic conditions are thought to be analogous to the fed, starved and re-fed state *in vivo* respectively. Long-chain acyl-CoA contents were 110±5, 203±22 and 113±7 nmol/g of protein respectively. Consistently, in the starved state the mitochondrial ATP/ADP ratio was increased and the cytosolic ratio was decreased compared with the fed state, and these changes were reversed by the addition of glucose and glycerol. Infusion of glucose and glycerol lowered the content of long-chain acyl-CoA, probably owing to increased esterification of fatty acids. The changes in subcellular ATP/ADP ratios were more pronounced in perfused liver than in liver *in vivo*. This is mostly due to the very low mitochondrial ATP/ADP ratio found in perfused liver in the fed state. As mentioned in a previous paper (Schwenke *et al.*, 1981), it may be caused by large oxygen gradients in haemoglobin-free perfused rat liver. This point, however, needs to be examined further.

In a second set of experiments livers from fed rats were perfused with oleate or octanoate in the presence of 2.0% (w/v) albumin (Table 2). Changes similar to those in the starved state were found

with oleate, although the decrease in the cytosolic ATP/ADP ratio was not significant. Octanoate as a short-chain fatty acid, however, had no effect on mitochondrial as well as cytosolic ATP/ADP ratios.

ATP/ADP ratios in isolated hepatocytes

In hepatocytes from fed rats fractionated with digitonin, mitochondrial ATP/ADP ratios were increased with increasing oleate concentrations up to 1 mM, consistent with the changes in long-chain acyl-CoA content; no significant changes occurred in the cytosolic ATP/ADP ratios in the same concentration range. At oleate concentrations higher than 1 mM the cytosolic ratio decreased, whereas the mitochondrial ratio and long-chain acyl-CoA content did not change (Fig. 1*a*). When albumin was lowered from 2.0 (Fig. 1*a*) to 0.2% (w/v) (Fig. 1*b*) at the same oleate concentrations, both mitochondrial and cytosolic ATP/ADP ratios decreased, although the long-chain acyl-CoA content increased with increasing oleate concentration even more than in the experiment with high albumin. A stimulation of oxygen uptake from 12 to 24 nmol of O_2/min per mg of protein was measured with 1 mM-oleate at 0.2% albumin and from 15 to 19 nmol of O_2/min per mg at 2% albumin.

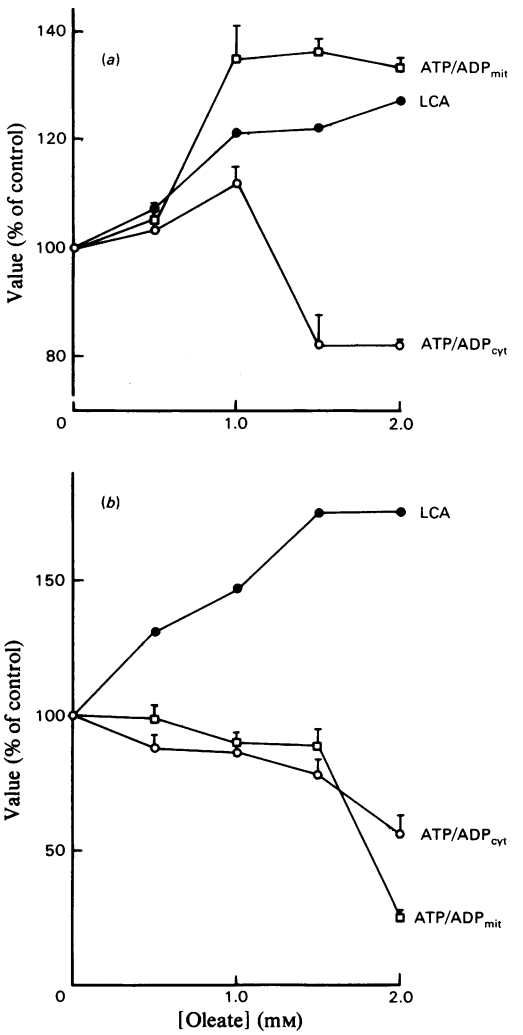

Fig. 1. *Effect of oleate on mitochondrial (mit) and cytosolic (cyt) ATP/ADP ratios in isolated hepatocytes from fed rats at different albumin concentrations*

(*a*) 2.0% albumin; (*b*) 0.2% albumin. Data are mean values ± S.E.M.; $n = 4$ for ATP/ADP ratios; $n = 2$ for long-chain acyl-CoA (LCA) measurements. Control values were: ATP/ADP$_{mit}$ 2.6 ± 0.8; ATP/ADP$_{cyt}$ 13.4 ± 4.5; LCA 138 nmol/g of protein. For incubation conditions see the Methods section.

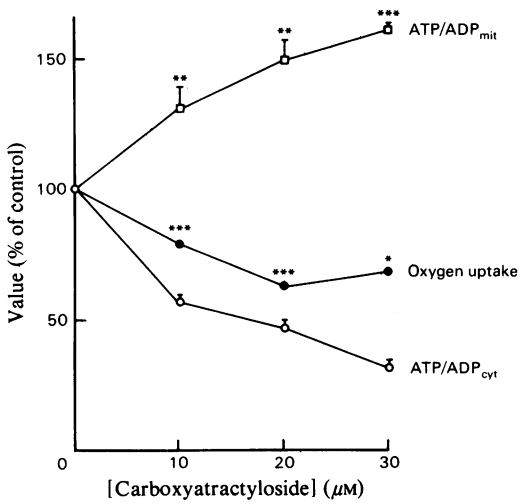

Fig. 2. *Effect of carboxyatractyloside on mitochondrial (mit) and cytosolic (cyt) ATP/ADP ratios in isolated hepatocytes from fed rats*

Data are mean values ± S.E.M.; $n = 4$ for ATP/ADP ratios; $n = 2$ for oxygen measurements. Control values were: ATP/ADP$_{mit}$ 1.9 ± 0.3; ATP/ADP$_{cyt}$ 10.3 ± 3.5; oxygen uptake 11.0 nmol/min per mg of protein. For incubation conditions see the Methods section; for key to symbols for *P* values, see Table 1.

With carboxyatractyloside, mitochondrial ATP/ADP ratios were increased and cytosolic ratios were decreased with increasing inhibitor concentration (Fig. 2), similar to the changes shown in Fig. 1(*a*). In contrast with oleate, oxygen uptake was decreased.

Discussion

When carboxyatractyloside is added to liver preparations, i.e. isolated hepatocytes or perfused liver (Zuurendonk & Tager, 1974; Soboll *et al.*, 1978; Fig. 2), the inhibition of mitochondrial aden-

ine nucleotide translocation is reflected by characteristic changes in the subcellular ATP systems: the mitochondrial ATP/ADP ratio increases and the cytosolic ratio decreases. Under metabolic conditions in liver which are characterized by an elevated tissue content of long-chain acyl-CoA, the change of the subcellular ATP/ADP ratios resembles closely that observed with carboxyatractyloside. This is demonstrated in the present paper (i) in livers *in vivo*, (ii) in perfused livers taken from fed and starved rats, (iii) in perfused livers from fed rats after the addition of oleate, but not octanoate, and (iv) in isolated hepatocytes incubated with oleate in the presence of 2% albumin (Fig. 1*a*), but not at low (0.2%) albumin (Fig. 1*b*). If not measured in the same preparation, sufficient experimental data are available showing that the tissue content of long-chain acyl-CoA was elevated in perfused liver and isolated hepatocytes after oleate addition (Williamson *et al.*, 1969; Akerboom *et al.*, 1977) or *in vivo* during starvation (McLean *et al.*, 1971; Seitz *et al.*, 1977).

The effect of exogenously added oleate on ATP/ADP ratios is seen in the mitochondrial compartment already at 1 mM-oleate, whereas the cytosol responds at 1.5 mM-oleate (Figs. 1*a* and 1*b*). This is probably due to the fact that oleate is metabolized, generating ATP, which is exported to the cytosol. Thus this so-called substrate effect may be superimposed on the inhibitory effect of oleoyl-CoA and free oleate, especially in the lower concentration range.

The mitochondrial changes are dependent on the albumin concentration added. At 2.0% albumin, an increase in mitochondrial ATP/ADP ratios with up to 1 mM-oleate is observed in isolated hepatocytes, which is consistent with an increase in long-chain acyl-CoA, whereas at higher oleate concentrations no further change is observed. At this high free fatty acid concentration the effect of uncoupling probably overlaps the inhibitory effect of long-chain acyl-CoA on adenine nucleotide translocation. Thus uncoupling prevents a further increase in ATP/ADP ratio. This is more clearly demonstrated in Fig. 1(*b*). At low (0.2%) albumin uncoupling occurs at oleate concentrations below 1 mM, preventing an increase in mitochondrial ATP/ADP ratio despite increased long-chain acyl-CoA. A marked decrease in mitochondrial as well as cytosolic ratios is observed at oleate concentrations higher than 1.5 mM. The onset of uncoupling in Fig. 1(*b*) even at low oleate concentrations is further indicated by the simultaneous large stimulation of oxygen uptake (100% with 0.2% albumin; 27% with 2.0% albumin), as demonstrated previously in isolated mitochondria (Lehninger & Remmert, 1959; Borst *et al.*, 1962; Bode & Klingenberg, 1965).

It may be argued that the alterations in mitochondrial and extramitochondrial ATP/ADP ratios are not induced by long-chain acyl-CoA, but are the consequence of fatty acid metabolism, e.g. stimulation of mitochondrial ATP synthesis by fatty acid substrate supply. However, the addition of the short-chain fatty acid octanoate to perfused liver failed to increase mitochondrial ATP/ADP ratios (Table 2).

The physiological significance of the long-chain acyl-CoA-mediated inhibition of adenine nucleotide translocase is suggested from the following observations. Oleate stimulates gluconeogenesis in hepatocytes only from precursors which require the pyruvate-carboxylation step, e.g. lactate, whereas gluconeogenesis from dihydroxyacetone is not affected (Brocks *et al.*, 1980). Moreover, stimulation of gluconeogenesis may be exerted via inhibition of pyruvate dehydrogenase and activation of pyruvate carboxylase, both mediated by the mitochondrial ATP/ADP ratio (Wieland & Weiss, 1963; Walter *et al.*, 1966). Consequently pyruvate, which is sparse in the starved state, is diverted from oxidation in the citric acid cycle to carboxylation for gluconeogenesis. Thus alterations in the mitochondrial ATP/ADP ratios as a consequence of inhibition/deinhibition of adenine nucleotide translocation by long-chain acyl-CoA may be, in fact, involved in the regulation of hepatic pyruvate metabolism.

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