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Inhibitory effects of some long-chain unsaturated fatty acids on mitochondrial β-oxidation

Effects of streptozotocin-induced diabetes on mitochondrial β-oxidation of polyunsaturated fatty acids

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1. Evidence showing that some unsaturated fatty acids, and in particular docosahexaenoic acid, can be powerful inhibitors of mitochondrial β-oxidation is presented. This inhibitory property is, however, also observed with the *cis*- and *trans*-isomers of the $C_{18:1(6)}$ acid. Hence it is probably the position of the double bond(s), and not the degree of unsaturation, which confers the inhibitory property. 2. It is suggested that the inhibitory effect is caused by accumulation of 2,4-di- or 2,4,7-trienoyl-CoA esters in the mitochondrial matrix. This has previously been shown to occur with these fatty acids, in particular when the supply of NADPH was limiting 2,4-dienoyl-CoA reductase (EC 1.3.1.–) activity [Hiltunen, Osmundsen & Bremer (1983) *Biochim. Biophys. Acta* **752**, 223–232]. 3. Liver mitochondria from streptozotocin-diabetic rats showed an increased ability to β-oxidize 2,4-dienoyl-CoA-requiring acylcarnitines. Docosahexaenoylcarnitine was also found to be less inhibitory at lower concentrations with incubation under coupled conditions. With uncoupling conditions there was little difference between mitochondria from normal and diabetic rats in these respects. This correlates with a 5-fold stimulation of 2,4-dienoyl-CoA reductase activity found in mitochondria from streptozotocin-diabetic rats.

Mitochondrial β-oxidation of fatty acids possessing double bonds at even-numbered carbon atoms, counted from the carboxy end of the molecule, require the activity of 2,4-dienoyl-CoA reductase (EC 1.3.1.–) for their oxidation (Osmundsen *et al.*, 1982; Hiltunen *et al.*, 1983). This activity converts the 2,4-dienoyl-CoA ester, which is generated during β-oxidation of these fatty acids, into a *trans*-Δ^3-enoyl-CoA ester (Dommes *et al.*, 1983). In the absence of an adequate supply of NADPH, 2,4-di- or 2,4,7-tri-enoyl-CoA β-oxidation intermediates can accumulate (Hiltunen *et al.*, 1983). For this reason uncoupling has been found to inhibit β-oxidation of polyunsaturated fatty acids, because the energy-dependent $NAD(P)^+$ transhydrogenase (EC 1.6.1.1) cannot supply the required amounts of NADPH (Osmundsen *et al.*, 1982). Similarly, β-oxidation of polyunsaturated fatty acids was stimulated by the addition of glutamate, presumably because this substrate directly can supply NADPH through the activity of glutamate dehydrogenase (EC 1.4.1.3) (Hiltunen *et al.*, 1983).

It can therefore be argued that polyunsaturated fatty acids have the potential to inhibit β-oxidation of fatty acids not requiring 2,4-dienoyl-CoA reductase for their β-oxidation. This inhibitory property should be most potent under conditions when the supply of intramitochondrial NADPH is limited, analogous to findings with pent-4-enoic acid (Borrebæk *et al.*, 1980). If the position of the double bond is correct, a similar inhibitory effect should also be expressed with a long-chain monounsaturated fatty acid.

These possibilities have been investigated by using isolated rat liver mitochondria. Evidence is presented suggesting that polyunsaturated fatty acids can indeed strongly inhibit β-oxidation of other fatty acids, and that this effect is also expressed with a monounsaturated fatty acid having its double bond at an even-numbered carbon atom. Also, in streptozotocin-diabetic rats, the 2,4-dienoyl-CoA reductase activity is shown to be increased, and low concentrations of the polyunsaturated fatty acids can now have a lesser

Abbreviation used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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inhibitory effect on β-oxidation of other fatty acids.

Experimental

Materials

Male Wistar albino rats were obtained from Veterinær Möllers Avlsstation, Havdrup, Denmark. When used in experiments the rats weighed 180–300g. Rat liver mitochondria were isolated as described by Osmundsen & Bremer (1977). EGTA, Mops, Hepes, ADP (grade X), ATP (grade I), CoA (grade IIIS), oleic acid, NADPH (type X), valinomycin, FCCP and streptozotocin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Docosahexaenoic acid, arachidonic acid, γ-linolenic acid, linoleic acid, petroselinic acid (*cis*-$C_{18:1(6)}$), petroselaidic acid (*trans*-$C_{18:1(6)}$) and vaccinic acid (*cis*-$C_{18:1(11)}$) were purchased from Nu Chek Prep, Elysian, MN, U.S.A. Palmitic acid and *NN'*-carbonyldi-imidazole (Puriss) were purchased from Fluka A.G., Buchs, Switzerland. *cis*-4-Decen-1-ol was purchased from K & K Laboratories, Plainview, NY, U.S.A., and converted into dec-4-enoic acid by oxidation with CrO_3/H_2SO_4 as described by Bowers *et al.* (1953). Dec-4-enoyl-CoA was synthesized and its concentration measured as described by Osmundsen *et al.* (1979).

[U-^{14}C]Palmitic acid was purchased from Amersham International, Amersham, Bucks., U.K., and [U^{14}C]palmitoylcarnitine was synthesized as described by Bremer & Wojtczak (1972). L-Carnitine was kindly given by Sigma Tau, Rome, Italy. All other acylcarnitines were synthesized as described by Červenka & Osmundsen (1982).

Rats were rendered diabetic by intraperitoneal injections of a single dose (100mg/kg body wt.) of streptozotocin, and 2 weeks later the rats were used in the experiments. Rats classified as diabetic had blood [glucose] of 18mM or higher.

Measurements of mitochondrial β-oxidation

Rates of oxidation of the various acylcarnitines were measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). The basic incubation medium contained 130mM-KCl, 10mM-Hepes, 0.1mM-EGTA, 5mM-$MgCl_2$, 2mM-P_i and 2mg of defatted bovine serum/ml, pH7.2. The incubations typically contained 2–3mg of mitochondrial protein in a final volume of 2.5ml. Acylcarnitines were included at a concentration of 40μm. The incubation medium also contained 5mM-malonate or 2mM-L-malate. To measure the inhibitory effect of an acylcarnitine, this was added 2–3min before addition of 40μM-oleoylcarnitine. The combined rate of respiration was, if an inhibitory effect was expressed, lower than that obtained with oleoylcarnitine alone. Reversing the order of addition generated a non-linear time-course of respiration, the rate of which decreased with increasing time of incubation. Uncoupling was always achieved by addition of 4μM-FCCP or 0.4nM-valinomycin before addition of acylcarnitines.

Rates of β-oxidation was also measured as acid-soluble [in 2.5% (v/v) $HClO_4$] radioactivity generated from [U-^{14}C]palmitoylcarnitine as described previously (Osmundsen & Bremer, 1977).

All incubations were carried out at 30°C. The pH of solutions was adjusted by addition of aq. KOH or HCl, or by additions of solid $KHCO_3$ or K_2CO_3.

Assay of mitochondrial protein

Mitochondrial protein was measured with the biuret assay (Gornall *et al.*, 1949), modified to contain 0.3% deoxycholate to ensure solubilization of mitochondrial proteins.

Assay of 2,4-dienoyl-CoA reductase activity in isolated rat liver mitochondria

Mitochondrial suspensions were frozen and thawed once, and the protein concentration was adjusted to 20mg/ml with 50mM-potassium phosphate buffer, pH7.2. To the diluted suspensions was added Triton X-100 (final concn. 0.5%, v/v). After stirring for 5min at 0°C, the lysed mitochondrial suspensions were centrifuged at 100000$g_{av.}$ for 30min. The resulting clear yellow supernatant was used for assay of 2,4-dienoyl-CoA reductase activity, as described by Kunau & Dommes (1978), with dec-4-enoyl-CoA as the substrate, at 100μM (this concentration was found to give maximal rates of activity). The assays were carried out at 30°C.

Statistical analysis

The statistical significance of differences between sets of experimental data was calculated by using the Bonferroni modification of the *t* test as described by Wallenstein *et al.* (1980): P value ≤ 0.05 was taken to indicate a significant difference.

Results

Effects of docosahexaenoylcarnitine on mitochondrial oxidation of oleoylcarnitine

Results in Fig. 1 show that addition of increasing concentrations of docosahexaenoylcarnitine to coupled mitochondrial incubations caused progressively decreased rates of oxidaton of subsequently added oleoylcarnitine. Docosahexaenoylcarnitine appeared to be a powerful inhibitor, as it, at about 7μM, caused about 50% inhibition in the

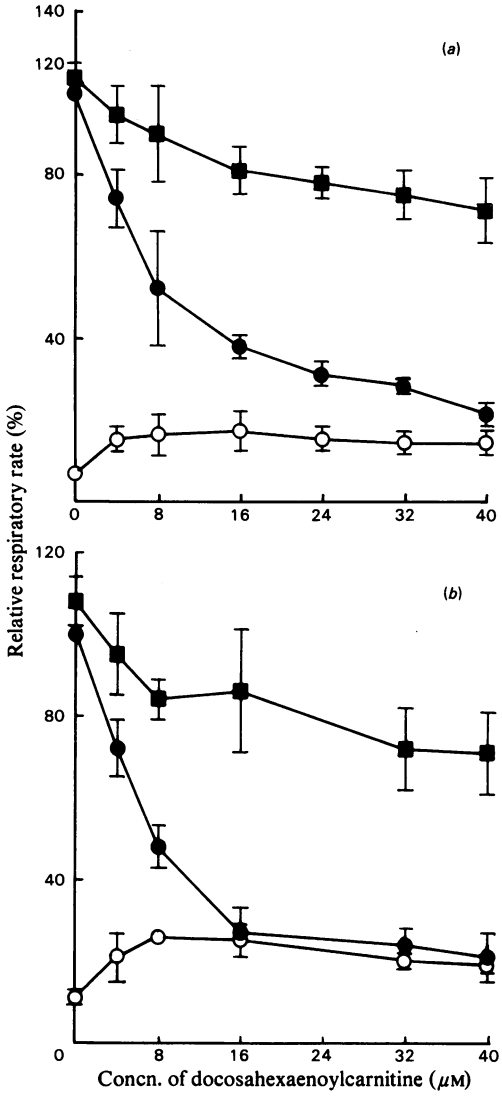

Fig. 1. *Effects of various concentrations of docosahexaenoylcarnitine on rates of respiration obtained together with subsequently added oleoylcarnitine with coupling conditions of incubation*

Rat liver mitochondria were incubated in the presence of 2mM-ADP, and then, with either 5mM-malonate (*a*) or 2mM-malate (*b*), docosahexaenoylcarnitine was added to the concentrations shown 2min before addition of 40μM-oleoylcarnitine. After about 4–5min, 5mM-glutamate was added to the incubation. The rates of respiration were measured before addition of oleoylcarnitine (○), after addition of oleoylcarnitine (●) and after addition of glutamate (■). Means ±S.E.M. for three experimental animals are shown. Respiratory rates are expressed as percentages of the rates obtained with oleoylcarnitine alone (set at 100%). The absolute rates obtained with oleoylcarnitine were 32±3 and 78±6ng-atoms of O/min per mg of protein in (*a*) and (*b*) respectively. Other details are given in the Experimental section.

presence of 5mM-malonate. At a concentration equimolar to that of oleoylcarnitine (40μM), almost 100% inhibition of the oleoylcarnitine-dependent respiratory rate resulted. This inhibitory effect was expressed whether 2mM-malate or 5mM-malonate was included in the incubation medium (see Fig. 1).

With uncoupling conditions the inhibitory effect of the docosahexaenoylcarnitine became even more marked. In the presence of malonate, oleoylcarnitine-dependent respiration was almost completely suppressed in the presence of 4μM-docosahexaenoylcarnitine. Increasing concentrations of this acylcarnitine caused no further inhibition (see Fig. 2*a*). With malate present in the incubations, however, this concentration of docosahexaenoylcarnitine caused only about 30% inhibition of oxygen uptake, and with equimolar concentrations of the two acylcarnitines about 60% inhibition resulted (see Fig. 2*b*).

Mitochondrial β-oxidation of polyunsaturated acylcarnitines has previously been shown to be stimulated by addition of glutamate (Hiltunen *et al.*, 1983). Even in the presence of 5mM-glutamate, however, an inhibitory effect of docosahexaenoylcarnitine on mitochondrial β-oxidation of subsequently added oleoylcarnitine was clearly observed (see Figs. 1 and 2).

The results presented in Fig. 3 show clearly that the effect of added docosahexaenoylcarnitine is to inhibit completely the oxidation of [U-^{14}C]palmitoylcarnitine. Addition of 5mM-glutamate caused only minor stimulation of oxidation of [U-^{14}C]-palmitoylcarnitine. Hence the stimulatory effect of glutamate on oxygen uptake is primarily caused by stimulated oxidation of docosahexaenoylcarnitine (apart from the smaller contribution from glutamate itself; see Hiltunen *et al.*, 1983).

Arachidonoylcarnitine showed a similar inhibitory effect on oleoylcarnitine-dependent respiration, although less pronounced than that of docosahexaenoylcarnitine. With uncoupling conditions of incubation, however, practically complete inhibition of oleoylcarnitine-dependent stimulation of respiration was achieved with arachidonoylcarnitine included at a concentration of 16μM, provided that the incubation medium also contained 5mM-malonate. When malonate was replaced with 2mM-malate, only about 25% inhibition was obtained (results not shown). With coupled conditions of incubation, in the presence of malate, no inhibitory effect of arachidonoylcarnitine was observed (results not shown).

Petroselinoylcarnitine and petroselaidoylcarnitine both showed inhibitory properties similar to those described for arachidonoylcarnitine (results not shown). The positional isomer with the double bond in the Δ^{11}-position (vaccinoylcarnitine),

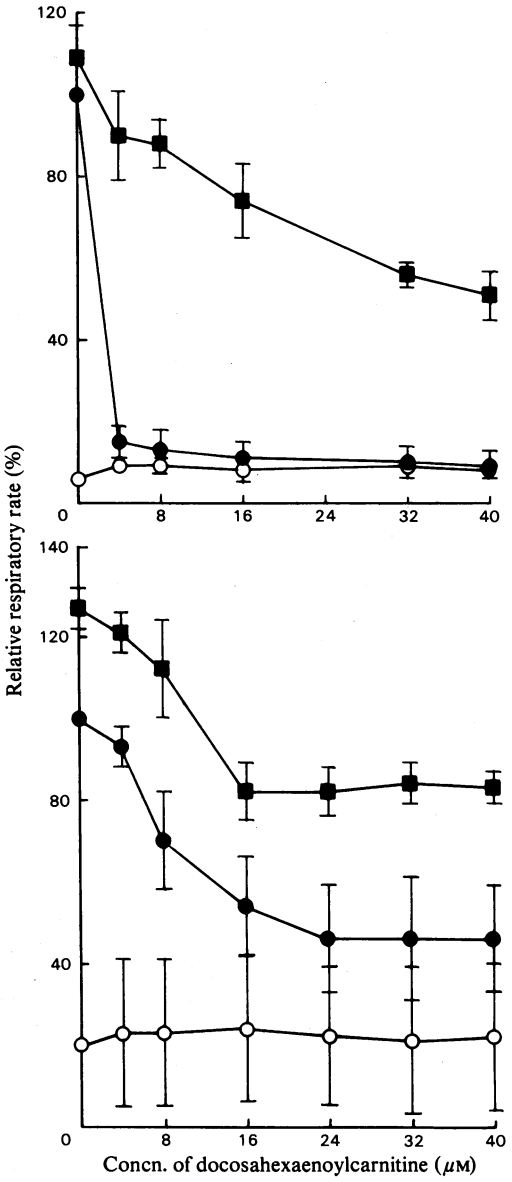

Fig. 2. *Effects of various concentrations of docosahexaenoylcarnitine on rates of respiration obtained together with subsequently added oleoylcarnitine with uncoupling conditions of incubation*

Rat liver mitochondria were incubated in the presence of 4mM-FCCP and either 5mM-malonate (*a*) or 2mM-malate (*b*), and then docosahexaenoylcarnitine, oleoylcarnitine and glutamate were added as described in the legend to Fig. 1. Rates of respiration were measured before addition of oleoylcarnitine (○), after addition of oleoylcarnitine (●) and after addition of 5mM-glutamate (■). The absolute rates of respiration obtained with oleoylcarnitine alone (set at 100%) were 41±6 and 110±10ng-atoms of O/min per mg of protein in (*a*) and (*b*) respectively.

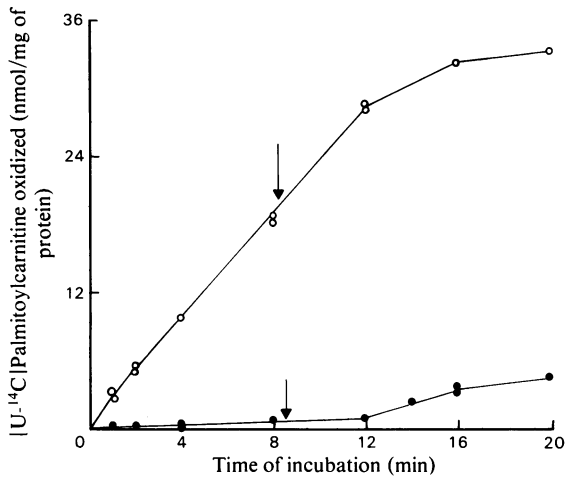

Fig. 3. *Effect of docosahexaenoylcarnitine on β-oxidation of [U-^{14}C]palmitoylcarnitine*

Rat liver mitochondria were incubated for 2min in the presence of 40μM-docosahexaenoylcarnitine, and then 40μM-[U^{14}C]palmitoylcarnitine (sp. radioactivity 850d.p.m./nmol) was added. The incubation also contained 5mM-malonate and 4μM-FCCP. The total incubation volume was 2.0ml. Samples (200μl) were removed at the time intervals shown for measurement of acid-soluble radioactivity (●). Identical incubations, not containing docosahexaenoylcarnitine, were sampled in the same fashion (○). The unlabelled arrows indicate time of addition of 5mM-glutamate to the incubations. Results obtained with duplicate incubations are shown. Each incubation contained about 2mg of mitochondrial protein. Further details are given in the Experimental section.

however, showed no inhibitory effect (results not shown), suggesting that the position of the double bond is significant for the inhibitory effect to be expressed.

Effects of streptozotocin-induced diabetes on β-oxidation of polyunsaturated fatty acids by isolated rat liver mitochondria

The ability of docosahexaenoylcarnitine to inhibit the oxidation of subsequently added oleoylcarnitine was also examined with mitochondria isolated from streptozotocin-diabetic rats. The results presented in Fig. 4 show that the inhibitory effect was then much smaller at lower concentrations of docosahexaenoylcarnitine, or even completely absent. In the presence of 5mM-malonate an equimolar concentration of docosahexaenoylcarnitine was required to suppress oleoylcarnitine-stimulated respiration extensively, provided that 2mM-ADP was present in the medium. With 4μM-FCCP in place of ADP, oleoylcarnitine-dependent stimulation of respiration was, as expected, much

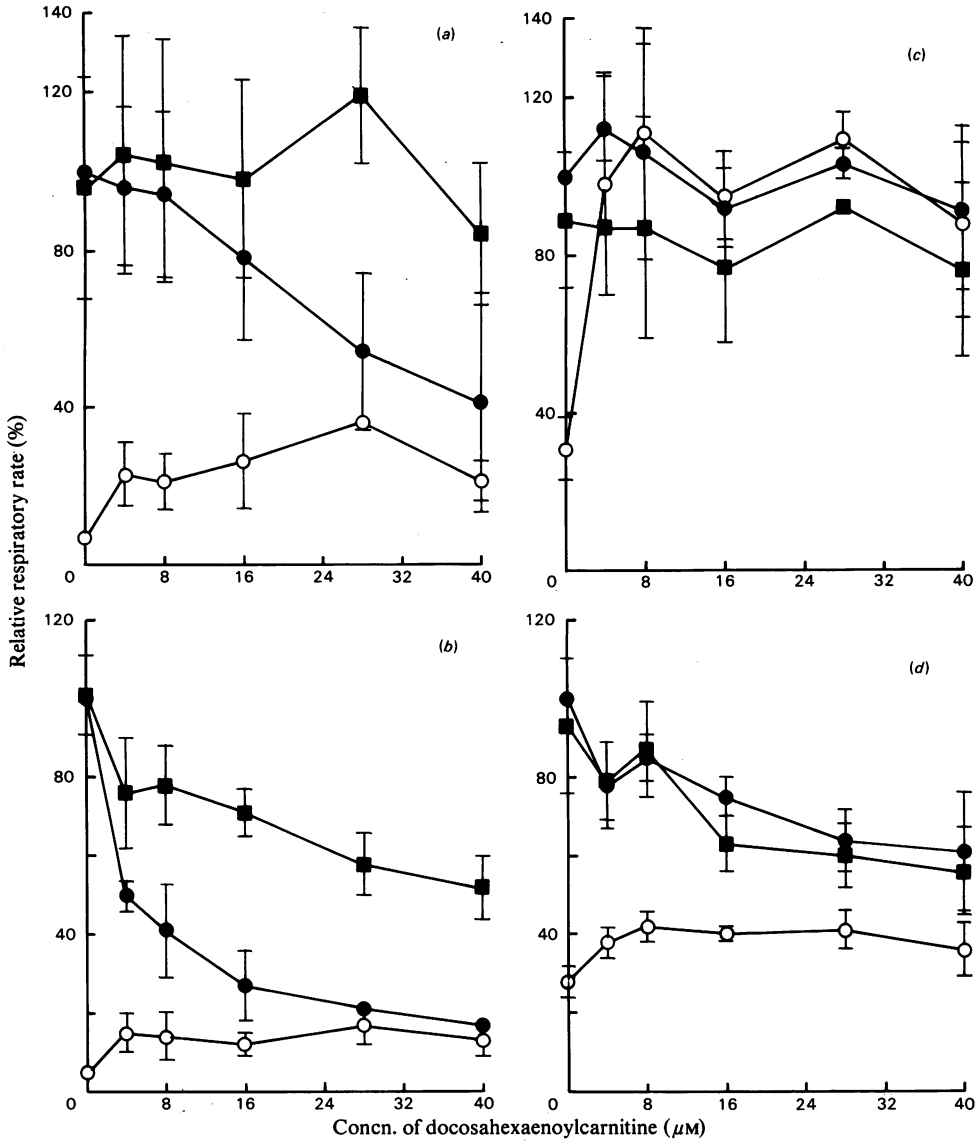

Fig. 4. *Effects of various concentrations of docosahexaenoylcarnitine on rates of respiration obtained together with oleoylcarnitine in liver mitochondria from streptozotocin-diabetic rats*

Mitochondria isolated from livers of rats (180g body wt.) made diabetic by intraperitoneal injections of streptozotocin (100mg/kg body wt.) were incubated in the presence of docosahexaenoylcarnitine, oleoylcarnitine and glutamate as described in the legend to Fig. 1. Rates of respiration were measured after addition of docosahexaenoylcarnitine (○), after addition of oleoylcarnitine (●) and after addition of glutamate (■). Incubations were carried out in the presence of 5mM-malonate and 2mM-ADP (*a*) or 4μM-FCCP (*b*), or in the presence of 2mM-malate and 2mM-ADP (*c*) or 4μM-FCCP (*d*). The results are means ± S.E.M. for three or four rats. Respiratory rates are expressed as percentages of the rates obtained with oleolycarnitine alone (set at 100%). The absolute rates obtained with oleolycarnitine were 36 ± 5, 41 ± 3, 68 ± 7 and 136 ± 12ng-atoms of O/min per mg of protein in (*a*), (*b*), (*c*) and (*d*) respectively.

more sensitive to inhibition. Then 16μM-docosahexaenoylcarnitine gave almost complete inhibition with mitochondria from diabetic rats (Fig. 4). With uncoupling conditions, therefore, the sensitivity of the β-oxidation system to inhibition by docosahexaenoylcarnitine is more similar to that observed with uncoupled normal mitochondria (see Fig. 1).

With malate and ADP in the incubation medium, no inhibitory effect of docosahexaenoylcarnitine was observed, and the rate of respiration observed with docosahexaenoylcarnitine was not further stimulated on addition of oleoylcarnitine (Fig. 4). In the presence of malate and FCCP, however, the inhibitory effect of docosahexaenoylcarnitine re-appeared, although complete inhibition of oleoylcarnitine-dependent stimulation of respiration was not achieved, even with 40 μM-docosahexaenoylcarnitine (Fig. 4).

Results presented in Table 1 show that streptozotocin-induced diabetes in relatively young rats (about 180g body wt.) can cause a selective increase in mitochondrial ability to β-oxidize the unsaturated acylcarnitines, except docosahexaenoylcarnitine and oleoylcarnitine. In somewhat older rats (about 300g body wt.) a general increase in rates of β-oxidation was observed for all acylcarnitines, although the control rates were not significantly altered (results not shown). This latter finding is in line with previous results regarding effects of diabetes on hepatic fatty acid oxidation, which have shown that rates of ketogenesis are increased, as is also the activity of carnitine palmitoyltransferase (Van Harken *et al.*, 1969; Hararo *et al.*, 1972). The enhanced ability to β-oxidize most of these unsaturated acylcarnitines (except oleoylcarnitine) is most probably caused by the significantly increased 2,4-dienoyl-CoA reductase activity measured in extracts from liver mitochondria in all diabetic rats. As measured with dec-4-enoyl-CoA as substrate, an activity of 5 ± 0.5 ($n = 4$) nmol of NADPH oxidized/min per

Table 1. *Effects of various unsaturated acylcarnitines on combined rates of respiration together with oleoylcarnitine, with mitochondria from livers of normal and diabetic rats*

Liver mitochondria isolated from normal rats, and from young (180g body wt.) and older (300g body wt.) streptozotocin-diabetic rats were incubated in the presence of 5mM-malonate and 2mM-ADP. Rates of respiration were measured after addition of 40 μM of an unsaturated acylcarnitine, after 40 μM-oleoylcarnitine was added 2min later, and after addition of 5mM-glutamate (added 4–5min after oleoylcarnitine). The reference incubations contained oleoylcarnitine and glutamate only (top of Table). Numbers of animals investigated are shown in parentheses, and the tabulated values are means ±S.E.M. Further details are given in the Experimental section. *Significant difference between means for control and corresponding diabetic; †significant differences between means for corresponding 'young' and 'old' diabetic rats; ‡significant stimulation/inhibition of respiratory rates after addition of glutamate.

	Rates of respiration (ng-atoms of O/min per mg of mitochondrial protein)		
Acylcarnitine(s)	Control	Young diabetic	Old diabetic
Oleoyl-	37 ± 7 (4)	43 ± 17 (4)	69 ± 10* (3)
+5mM-glutamate	28 ± 1 (4)	37 ± 7* (4)	54 ± 14*† (3)
Dec-4-enoyl-	41 ± 6 (3)	67 ± 13* (4)	87 ± 5*† (3)
+oleoyl-	37 ± 3 (3)	54 ± 10* (4)	60 ± 16* (3)
+glutamate	35 ± 8 (3)	45 ± 12 (4)	33 ± 8‡ (3)
Linoleoyl-	35 ± 7 (3)	49 ± 15 (4)	72 ± 16* (3)
+oleoyl-	37 ± 7 (3)	48 ± 16 (4)	65 ± 15* (3)
+glutamate	34 ± 3 (3)	38 ± 7 (4)	37 ± 7‡ (3)
γ-Linolenoyl-	7 ± 1 (3)	20 ± 7* (4)	29 ± 10* (3)
+oleoyl-	18 ± 5 (3)	31 ± 9* (4)	51 ± 1*† (3)
+glutamate	31 ± 1‡ (3)	34 ± 4 (4)	41 ± 8* (3)
Arachidonoyl-	8 ± 2 (3)	14 ± 2* (4)	19 ± 7* (3)
+oleoyl-	26 ± 3 (3)	30 ± 4 (4)	52 ± 15*† (3)
+glutamate	29 ± 3 (3)	28 ± 4 (4)	42 ± 10*†‡ (3)
Docosahexaenoyl-	7 ± 2 (3)	8 ± 1 (4)	12 ± 7 (3)
+oleoyl-	15 ± 1 (3)	18 ± 5 (4)	45 ± 16*† (3)
+glutamate	29 ± 2‡ (3)	28 ± 3‡ (4)	44 ± 10*† (3)
Petroselinoyl-	18 ± 5 (3)	26 ± 4* (4)	47 ± 9*† (3)
+oleoyl-	27 ± 6 (3)	32 ± 5 (4)	63 ± 14*† (3)
+glutamate	29 ± 2 (3)	30 ± 1 (4)	43 ± 5*†‡ (3)
Petroselaidoyl-	15 ± 7 (3)	25 ± 1* (4)	33 ± 9* (3)
+oleoyl-	27 ± 8 (3)	31 ± 1 (4)	51 ± 4*† (3)
+glutamate	28 ± 5 (3)	28 ± 3 (4)	38 ± 8*†‡ (3)

mg of protein was found with normal rats, whereas diabetic rats gave a value of 16 ± 3 ($n = 5$). No significant difference was observed between young and old diabetic rats in this respect.

Results presented in Table 1 also show that significant stimulation of respiration after addition of 5mM-glutamate was only observed with γ-linolenoylcarnitine and with docosahexaenoylcarnitine. With young diabetic rats this was only observed with docosahexaenoylcarnitine, whereas glutamate with mitochondria from old diabetic rats caused a significant decrease in rates of respiration with all acylcarnitines, except oleoylcarnitine. The reason for this is not clear.

With uncoupling conditions the enhanced ability to oxidize most of these unsaturated acylcarnitines vanished. Significant residual stimulation was still apparent with dec-4-enoylcarnitine and linoleoylcarnitine (results not shown). Oleoylcarnitine, which does not require 2,4-dienoyl-CoA reductase during its β-oxidation, gave, as expected, an increased respiratory rate also with uncoupling conditions (results not shown).

Some of the differences between young and old diabetic rats described here can be explained by a relatively selective increase in 2,4-dienoyl-CoA reductase activity in the young rats. In the older rats this activity is also enhanced, and in addition a marked general stimulation of β-oxidation occurred.

Enhanced ability to oxidize polyunsaturated fatty acids may be the reason why Vidal *et al.* (1983) observed an increased fraction of more-saturated fatty acids in the phospholipids derived from liver mitochondria of streptozotocin-diabetic rats.

Effects of valinomycin on docosahexaenoylcarnitine-dependent inhibition of β-oxidation of oleoylcarnitine or [U-^{14}C]palmitoylcarnitine, by isolated rat liver mitochondria

Uncoupling by means of FCCP, which brings about the collapse of the proton gradient across the mitochondrial inner membrane, consistently increased the inhibitory effect of the various acylcarnitines found to inhibit oleoylcarnitine-dependent respiration, as demonstrated by results presented above. For this reason it was decided to investigate whether the removal of the electrostatic charge component of the protonmotive force across the mitochondrial inner membrane, as achieved with valinomycin, would have a similar potentiating effect. Results presented in Fig. 5 show that valinomycin-stimulated respiration of oleoylcarnitine also was inhibited by addition of docosahexaenoylcarnitine, 8μM of which caused about 60% inhibition of oxidation of subsequently added

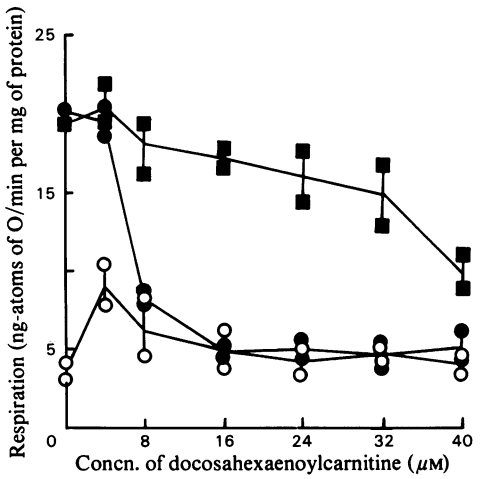

Fig. 5. *Effects of various concentrations of docosahexaenoylcarnitine on rates of respiration obtained together with oleoylcarnitine, in the presence of valinomycin*

Rat liver mitochondria were incubated in the presence of 5mM-malonate and 0.4nM-valinomycin. Docosahexaenoylcarnitine, oleoylcarnitine and glutamate were added as described in the legend to Fig. 1. Rates of respiration observed after addition of docosahexaenoylcarnitine (○), after addition of oleoylcarnitine (●) and after addition of glutamate (■) were measured.

oleoylcarnitine, provided that the incubation medium also contained 5mM-malonate. In the presence of 5mM-glutamate this inhibitory effect was still apparent, although much less marked (Fig. 5). With 2mM-malate included in the incubation medium (in place of 5mM-malonate), no inhibitory effect of docosahexaenoylcarnitine on valinomycin-stimulated respiration of oleoylcarnitine was apparent (results not shown).

Even in the presence of malate, however, the oxidation of the non-inhibitory acylcarnitine was inhibited. This is shown by results presented in Fig. 6, where the oxidation of [U-^{14}C]palmitoylcarnitine was monitored. From these results it is quite clear that the oxidation of [U-^{14}C]palmitoylcarnitine is more inhibited during valinomycin-stimulated respiration (by about 80%), as compared with ADP-stimulated oxidation (by about 55%). Hence it can be concluded that the relatively mild uncoupling, as achieved by using valinomycin, enhances the inhibitory effect of docosahexaenoylcarnitine. These results also suggest that a major fraction of the oxygen uptake observed in the presence of both docosahexanenoylcarnitine and oleoylcarnitine is derived from oxidation of the polyunsaturated acylcarnitine, in particular when malate is present in the incubation medium.

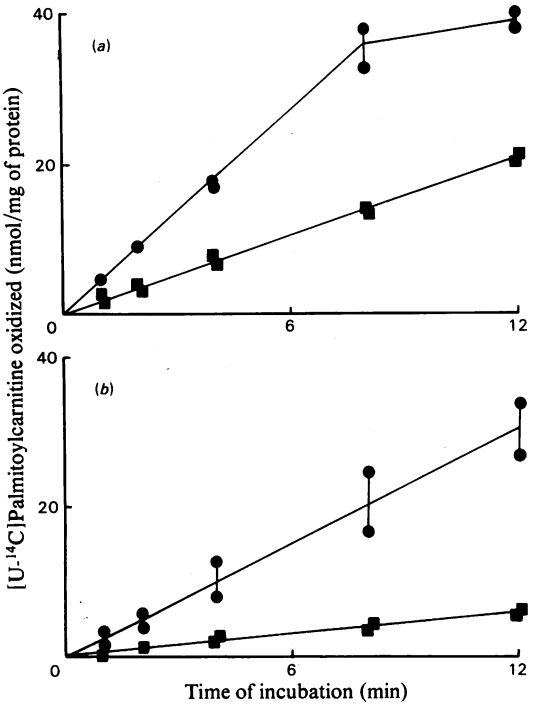

Fig. 6. *Effects of docosahexaenoylcarnitine on valinomycin-stimulated β-oxidation of [U-^{14}C]palmitoylcarnitine*
To rat liver mitochondria incubated in the presence of 2mM-malate and 2mM-ADP (*a*) or with 2mM-malate and 0.4nM-valinomycin (*b*) was added 40μM-docosahexaenoylcarnitine 2min before addition of 40μM-[U-^{14}C]palmitoylcarnitine. The total incubation volume was 1.5ml, and 200μl samples were removed at the times indicated (after addition of the last acylcarnitine) for measurement of acid-soluble radioactivity. The results shown are derived from duplicate experiments with incubations containing both acylcarnitines (■) and control incubations containing [U-^{14}C]palmitoylcarnitine as the sole carnitine ester (●). Further details are given in the Experimental section, and in the legend to Fig. 3.

Discussion

Mechanism of inhibition of mitochondrial β-oxidation by some unsaturated acylcarnitines

The inhibitory effect of the various acylcarnitines investigated here was always found to be potentiated by uncoupling. Their mechanism of action is therefore very different from that of other long-chain monounsaturated fatty acids, e.g. erucic acid or cetoleic (*cis*-$C_{22:1(11)}$) acid. These acyl-CoA esters are thought to impede the flux of shorter acyl-CoA esters across the acyl-CoA dehydrogenases (for review, see Bremer & Norum, 1982), and this inhibition is not influenced by uncoupling (Christophersen & Christiansen, 1975).

The present studies have shown that the position of the double bond(s) is significant for expression of an inhibitory effect. It is therefore clear, in the light of previous studies (Osmundsen *et al.*, 1982; Hiltunen *et al.*, 1983), that these acylcarnitines are inhibitory because their β-oxidation requires 2,4-dienoyl-CoA reductase activity. The 2,4-di- or 2,4,7-tri-enoyl-CoA ester, shown to accumulate in the mitochondrial matrix on addition of polyunsaturated acylcarnitine to rat liver mitochondria (Hiltunen *et al.*, 1983), is the probable source of the inhibitory effect. In the absence of an adequate supply of NADPH this β-oxidation intermediate cannot be further oxidized, presumably because it is a poor substrate for the enoyl-CoA hydratase (EC 4.2.1.17). In consequence, β-oxidation of fatty acids, which do not require participation of 2,4-dienoyl-CoA reductase for their β-oxidation, is also inhibited. This is probably due to competition with accumulated 2,4-di- or 2,4,7-tri-enoyl-CoA esters for access to the enoyl-CoA hydratase.

Such a mechanism of inhibition would readily explain the potentiating effect of uncoupling on inhibition, because uncoupling would remove the only NADPH source available in mitochondria incubated in the presence of malonate [that generated by NAD(P)$^+$ transhydrogenase]. In the presence of malate, however, the citric acid cycle is operating, and a supply of NADPH should be available from the NADP$^+$-dependent isocitrate dehydrogenase (EC 1.1.1.42). This is probably the reason why the inhibitory effect often decreased with malate present in the incubation, even in the presence of uncoupler (see Figs. 1 and 2).

The various unsaturated acylcarnitines exhibit different inhibitory potencies

Of the various acylcarnitines tested, docosahexaenoylcarnitine was by far the most potent inhibitor (see Table 1). The $C_{18:1(6)}$ isomers were equipotent with arachidonoylcarnitine, whereas γ-linolenoylcarnitine was second only to docosahexaenoylcarnitine.

With diabetic rats all of these, except docosahexaenoylcarnitine, were of similar inhibitory potency, although also docosahexaenoylcarnitine became much less inhibitory with mitochondria from older diabetic rats (Table 1). The reason for these differences is not entirely apparent. Docosahexaenoylcarnitine, however, possesses a double bond already before initiation of β-oxidation. Hence the inhibitory acyl-CoA species is formed without the acyl group having to complete one β-oxidation cycle. The other inhibitory acylcarnitines mentioned above require at least one cycle of β-oxidation before any 2,4-dienoyl-CoA ester is formed. Arachidonoyl-CoA, however, required two cycles for β-oxidation before formation of an

inhibitory acyl-CoA ester. It is therefore possible that fatty acids which form a 2,4-dienoyl-CoA ester at an early stage during their β-oxidation also are the most potent potential inhibitors of β-oxidation. This conclusion, however, is evidently not valid as regards dec-4-enoylcarnitine, which was not found to be inhibitory to oxidation of oleoylcarnitine. Pent-4-enoic acid, by contrast, is a potent inhibitor of β-oxidation (Holland & Sherratt, 1973). Cuebas & Schultz (1982) have, however, reported that *trans,trans*-deca-2,4-dienoyl-CoA is readily oxidized by isolated heart mitochondria, whereas the *cis,cis* or *cis,trans* isomers were not. This suggests that a major fraction of the dec-4-enoic acid is the *trans*-isomer.

Possible metabolic implications of polyunsaturated-fatty-acid-mediated inhibition of mitochondrial β-oxidation

During normal hepatic metabolism it is unlikely that any inhibitory effect of polyunsaturated fatty acids on mitochondrial β-oxidation is expressed. Rat liver mitochondria contain appreciable amounts of $NADP^+$, constituting about 60% of mitochondrial nicotinamide nucleotides (Glock & McLean, 1956), with a likely abundance of NADPH (for review, see Sies, 1982).

Reversible uncoupling has been shown to take place at low O_2 partial pressure (Kramer & Pearlstein, 1983). This phenomenon could be of physiological significance as regards β-oxidation of polyunsaturated fatty acids, if the supply of mitochondrial NADPH from alternative sources is simultaneously diminished, e.g. by reductive synthesis of glutamate via glutamate dehydrogenase (Tischler *et al.*, 1977). The apparent K_m of the 2,4-dienoyl-CoA reductase has been estimated as about 0.1 mM (Dommes *et al.*, 1983). It is therefore possible that the activity of the enzyme may be limited by changes in the [NADPH]/[$NADP^+$] ratio. The relatively mild uncoupling by valinomycin was in the present investigation found to potentiate the inhibitory effect, in line with this argument.

One might speculate that inhibition of β-oxidation mediated by a similar mechanism is significant as regards the etiology of Reye's syndrome (Reye *et al.*, 1963), which is symptomatically very similar to hypoglycin poisoning (Lowry, 1975). A metabolite of hypoglycin is known to cause partial inhibition of mitochondrial β-oxidation (Osmundsen & Sherratt, 1975), and also to interfere with β-oxidation of polyunsaturated fatty acids (Kunau & Lauterbach, 1978). Similarly, Thayer (1984) has suggested pent-4-enoic acid-dependent inhibition of β-oxidation as a model for Reye's syndrome.

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