Oxidation of *cis*-5-unsaturated fatty acids in intact rat liver mitochondria: the operation of reduction pathways

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The metabolism of *cis*-5 unsaturated fatty acids was studied in intact rat liver mitochondria to assess the operation of a reduction pathway. By using direct quantification of metabolites with a capillary-column gas chromatograph, 3-hydroxydodecanoate was identified among other metabolites when *cis*-5-dodecenoate was metabolized in intact rat liver mitochondria. The formation of 3-hydroxydodecanoate supports the existence of a reduction pathway in the metabolism of *cis*-5-unsaturated fatty acids. This metabolite cannot be produced from the conventional isomerase-mediated pathway. However, the data also indicated the possible operation of the conventional isomerase-mediated pathway in

intact rat liver mitochondria. The reduction pathway appears to account for at least 61% of the pathway for *cis*-5-dodecenoate. This reduction pathway was likely to proceed from the dehydrogenation to *trans*-2,*cis*-5-dodecadienoyl-CoA, which was isomerized to Δ^3,Δ^5 -dodecadienoyl-CoA, then to *trans*-2,*trans*-4-dodecadienoate. The reduction was mediated by 2,4-dienoyl-CoA reductase by the conversion of *trans*-2,*trans*-4-dodecadienoyl-CoA into *trans*-3-dodecenoyl-CoA. However, direct reduction of the *cis*-5 double bond was also shown to be operating, although to a lesser extent.

INTRODUCTION

The oxidation of the odd-numbered double bond in unsaturated fatty acids has been postulated to go through β -oxidation exclusively to a *cis*-3-enoyl-CoA, which was then isomerized to *trans*-2-enoyl-CoA by an isomerase [1]. Using enzyme preparations from rat liver mitochondria, we have shown the existence of a NADPH-dependent reduction pathway in the oxidation of *cis*-5-unsaturated fatty acids [2]. We proposed a direct reduction of the *cis*-5 double bond to saturated fatty acids to account for the mechanism of reduction. However, the reduction step can also occur in any of the intermediates after dehydrogenation by acyl-CoA dehydrogenases. In fact, Schulz and colleagues [3,4] recently showed that the reduction actually occurred at the step of *trans*-2,*trans*-4-dienoyl-CoA, which was produced from the isomerization of *trans*-2,*cis*-5-dienoyl-CoA through multiple steps.

Together with the requirement for 2,4-dienoyl-CoA reductase for the metabolism of even-numbered double-bond unsaturated fatty acids, the revised pathways for all unsaturated fatty acids appear to require a NADPH-dependent reduction step. Although these reduction reactions have been documented by using purified enzymes or crude enzyme preparations [2–8], the proof of the involvement of NADPH-dependent pathways in intact organelles has been more difficult. In *Escherichia coli*, the essential function of the reduction pathway has been demonstrated by using a mutant with a defective 2,4-dienoyl-CoA reductase [9]. However, no direct evidence has been obtained in mammalian systems. A number of investigators [10,11] have used indirect approaches to imply the involvement of NADPH in the metabolism of even-numbered double bonds in intact rat liver mitochondria. In their approaches, the addition of rotenone or glutamate stimulated the oxidation, whereas uncoupling with dinitrophenol in the

presence of malonate inhibited the oxidation, of certain long-chain polyunsaturated fatty acids. Taken together, these data were regarded as evidence that NADPH-producing machinery from isocitrate dehydrogenase, glutamate dehydrogenase and energy-dependent transhydrogenase were involved in the oxidation of even-numbered double-bond fatty acids.

The interpretation of the oxygen-consumption data in the presence of a number of potential substrates is not straightforward. In addition, substrates with various chain lengths and combinations of double bonds were used in these studies; this further complicates the interpretation. In the present investigation, well-defined substrates, i.e. *cis*-5-dodecenoic acid and dodecanoic acid, were used for comparison with a direct study of the metabolism using GLC analysis of metabolites. The metabolism of *cis*-4-decenoic acid was also studied for comparison. These direct pieces of evidence establish unequivocally the involvement of a reductive pathway for all unsaturated fatty acids in intact mitochondria.

MATERIALS AND METHODS

Chemicals

cis-5-Decenoic, *cis*-5-dodecenoic, *cis*-5-tetradecenoic and *cis*-4-decenoic acids were synthesized by the methods described previously [12]. Dodecanoic acid (lauric acid) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Acyl-CoAs were synthesized by the procedure of Goldman and Vegelos [13]. Their purity and concentration were determined by measuring the fatty acid released after alkaline hydrolysis. The identity and the amount of fatty acid released were determined with a dual capillary column gas chromatograph. In these preparations, no non-esterified

Abbreviation used: DNP, dinitrophenol.

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fatty acid was detectable before alkaline hydrolysis. In addition, the purity was also determined by the absorption spectra and the concentration determined by Ellman's method [14]. ATP, ADP, CoA (lithium salt), NADPH, dinitrophenol, clofibrate and rotenone were obtained from Sigma (St. Louis, MO, U.S.A.). [2,2'- $^2\text{H}_2$]*cis*-5-Dodecenoic acid was obtained by isotope exchange of *cis*-5-dodecenoic acid with $^2\text{H}_2\text{O}$ catalysed by methoxide ion as described previously [15].

Isolation of rat liver mitochondria

Sprague-Dawley rats (250–300 g) were fed with regular Purina rat chow. Fresh rat liver was minced and then homogenized in a mixture of MSM buffer (220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4) with EDTA (2 mM). After centrifugation for 10 min at 400 *g*, the homogenate was further centrifuged for 10 min at 7000 *g*. After two washes of the resulting pellets with MSM buffer, the mitochondria were suspended in this buffer to about 70 mg of protein/ml. The protein concentration was determined by the biuret method [16]. For the isolation of purified mitochondria, the mitochondria preparation from the above procedure was further purified in a self-forming 50 % Percoll gradient made isotonic with sucrose. After centrifuging at 79000 *g* for 30 min in a vertical rotor (Beckman VTi 50), the gradient was collected in 3 ml fractions. The mitochondrial fractions (density 1.08–1.10 g/ml) were located by using citrate synthase activity, and the peroxisomes (density 1.04–1.05 g/ml) were located by catalase activity [15]. Protein concentrations were determined by the Lowry method [17]. The purified mitochondria were obtained by diluting mitochondrial fraction of density 1.10 g/ml with 9 vol. of MSM buffer and centrifuging at 32000 *g* for 10 min.

Mitochondrial respiration study with oxygen electrode

The polarographic 'oxygen electrode' technique for measuring changes in oxygen utilization by mitochondria was carried out in a 1 ml chamber at 30 °C. A mixture of 1 mg of mitochondrial protein, MgCl_2 (1.6 μmol), ATP (1.6 μmol), CoA (0.9 μmol), L-carnitine (0.3 μmol), and malate (2.5 μmol) or malonate (5 μmol), in 1 ml of incubation mixture (80 mM KCl, 50 mM Mops, 5 mM P_i , 1 mM EGTA and 1 mg of defatted dialysed BSA/ml) was preincubated in the chamber. The final pH was 7.4. After depletion of endogenous substrates with a small amount of ADP, *cis*-5-dodecenoic acid (96 μM) was added and the oxygen consumption rates were measured. When acyl-CoAs (60 μM) were used as substrate cofactors (ATP, CoA, and MgCl_2) were omitted from the mixture, and the 'State 3' oxygen consumption rate was initiated by addition of ADP (0.2 mM) or dinitrophenol (50 μM). Only mitochondrial preparations with respiratory control ratio (RCR) larger than 6 (glutamate as substrate) were used for the studies.

Metabolic incubation studies

The studies were carried out in 25 ml Erlenmeyer flasks in a metabolic shaking incubator (140 cycles/min) at 37 °C with the same composition of incubation mixture, cofactors and substrates. The mitochondrial protein was 1 mg/ml and the total volume was 3 ml. A linear relationship in the disappearance of initial substrate was obtained at least between 5 and 60 min of incubation. Therefore, all data were obtained from a single-point study after 10 min of incubation. In some studies, rotenone (4 μM) was added and incubated for 1 min before the addition of substrates.

Metabolic profiling of incubation mixture

A sample (1 ml) of the incubation mixture was mixed with internal standard (20 μg of pentadecanoic acid) and hydrolysed at 25 °C for 45 min with the addition of 100 μl of 3 M KOH. The mixture was acidified and extracted with ethyl acetate/diethyl ether (1:1, v/v). After conversion into trimethylsilyl derivatives, the sample was analysed with a dual capillary column gas chromatograph, with a bonded dimethylpolysiloxane phase (SPB-1, from Supelco, Bellefonte, PA, U.S.A.) and a bonded 35 % diphenyl:65 % dimethylpolysiloxane phase (SPB-35) fused-silica capillary column. The column temperature was programmed to increase at 4 °C/min from 60 °C to 250 °C and a 50:1 split injection ratio. The quantification was based on the relative peak areas of sample and internal standard. The amount of metabolites was calculated as the weight equivalent to that of internal standard on the basis of area ratio.

A Hewlett-Packard 5985B gas chromatograph/mass spectrometer was used for metabolite identification. A shorter (12.5 m) fused-silica capillary column (SPB-1) was used with the same temperature program as described above for GC. The identities of the metabolites have been established in previous publications [2,15].

Selected ion monitoring determination of isotopic enrichments in metabolites

Derivatives of samples were separated by GLC through a capillary column of SPB-1 as described in the previous section. The column temperature was at 140 °C initially, then increased at a rate of 4 °C/min after injection. The chromatographic effluent was monitored at *m/z* 257, 258 and 259 for d_0 , d_1 and d_2 labels at a dwell time of 100 ms for each ion for dodecanoic acid. Peak areas were determined and normalized to the peak area of unlabelled ion (as 100). Metabolites produced from the incubation of unlabelled substrate were also analysed to obtain natural abundance isotope enrichments. For the calculation of percentage enrichment of each labelled species, the relative peak areas were corrected for the natural isotope abundance contribution by the technique described previously [18].

Reductase activities measured by the spectrophotometric method

This method is similar to the method described for the measurement of 2,4-dienoyl-CoA reductase activities [19]. Oxidation of NADPH monitored as the decrease in A_{340} was taken as the measure for the activities of reductases, with *cis*-5-dodecenoyl-CoA (30 μM) or *trans*-2,*trans*-4-decadienoyl-CoA (30 μM) as substrates. The activities were corrected for the blanks determined before the addition of substrates.

RESULTS

Oxygen-consumption rates of *cis*-5-dodecenol acid and related substrates in rat liver mitochondria

As shown in Table 1, the oxidation rate of *cis*-5-dodecenoyl-CoA was significantly lower than that of dodecanoyl-CoA when the studies were done in the presence of malonate. The oxidation rates in the presence of malate also appear to be lower for *cis*-5-dodecenoyl-CoA than for dodecanoyl-CoA; however, the differences are not statistically significant. Similarly to those reported by other investigators [10], the dinitrophenol (DNP)-stimulated rates for unsaturated substrates were significantly lower than the ADP-stimulated rates in the presence of malonate. The decreased oxygen-consumption rate observed in the DNP-uncoupled condition in the presence of malonate is consistent

Table 1 Oxygen-consumption rates of *cis*-5-dodecenoyl-CoA and dodecanoyl-CoA in rat liver mitochondria under ADP- and DNP-induced State-3 conditions

The studies were carried out in a 1 ml chamber at 30 °C. A mixture of 1 mg of mitochondrial protein, L-carnitine (0.3 μ mol), and malate (2.5 μ mol) or malonate (5 μ mol), was incubated in 1 ml of incubation mixture. ADP (0.2 mM) or DNP (50 μ M) was added to induce State 3 rate of acyl-CoA (60 μ M) oxidation. Data are expressed as means $\pm$ S.D.: * paired *t* test between ADP and DNP treatments; † paired *t* test between c5MC12-CoA and MC12-CoA. Abbreviations: ns, not significant; c5MC12-CoA, *cis*-5-dodecenoyl-CoA; MC12-CoA, dodecanoyl-CoA; c4MC10-CoA, *cis*-4-decenoyl-CoA; c5MC10-CoA, *cis*-5-decenoyl-CoA; MC16-CoA, palmitoyl-CoA; c5MC14-CoA, *cis*-5-tetradecenoyl-CoA; MC14-CoA, tetradecanoyl-CoA.

Substrate	Oxygen-consumption rate (ng-atoms/min per mg of protein)					
	Malate			Malonate		
	ADP	DNP	<i>P</i> *	ADP	DNP	<i>P</i> *
c5MC12-CoA	74 $\pm$ 18 (3)	82 $\pm$ 14 (34)	ns	43 $\pm$ 9 (5)	33 $\pm$ 8 (5)	0.01
MC12-CoA	92 $\pm$ 34 (3)	108 $\pm$ 24 (3)	ns	57 $\pm$ 11 (5)	50 $\pm$ 15 (5)	0.05
<i>P</i> †	ns			0.003		
c4MC10-CoA				30	11	
c5MC10-CoA				44	25	
MC16-CoA				44	25	
c5MC14-CoA				48	32	
MC14-CoA				50	37	

Table 2 Metabolites found from the incubation of *cis*-5-dodecenoyl-CoA in rat liver mitochondria under various conditions

The studies were carried out at 37 °C in a shaking incubator for 10 min. The incubation mixture (3 ml) contained mitochondria (3 mg of protein), *cis*-5-dodecenoyl-CoA (60 μ M), L-carnitine (0.3 mM) and malate (2.5 mM). ADP (0.2 mM), DNP (50 μ M), or rotenone (4 μ M) was added. Abbreviations: 3OHMC12, 3-hydroxydodecanoate; 3OHMC10, 3-hydroxydecanoate; 3OHMC8, 3-hydroxyoctanoate; t2t4MC12, *trans*-2,*trans*-4-dodecadienoate; 3,5MC12, combined amount of *trans*-3,*cis*-5- and *trans*-3,*trans*-5-dodecadienoate; 3OHc5MC12, 3-hydroxy-*cis*-5-dodecenoate. Data are expressed as means $\pm$ S.D.

Conditions	Concn. of metabolites (μ M)					
	3OHMC12	3OHMC10	3OHMC8	t2t4MC12	3,5MC12	3OHc5MC12
ADP (<i>n</i> = 4)	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	2.4 $\pm$ 2.2	1.3 $\pm$ 0.9	0 $\pm$ 0
DNP (<i>n</i> = 3)	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	2.9 $\pm$ 0.8	2.1 $\pm$ 1.1	0.3 $\pm$ 0.3
Rotenone (<i>n</i> = 4)	3.1 $\pm$ 0.7	4.4 $\pm$ 0.5	2.2 $\pm$ 1.0	4.1 $\pm$ 2.9	1.3 $\pm$ 1.6	2.0 $\pm$ 0.6

with an energy- and NADPH-dependent process in the metabolism of *cis*-5-dodecenoic acid. However, the oxygen-consumption rates for dodecanoic acid under identical conditions were also decreased, even though to a smaller extent. The oxidation of dodecanoic acid does not require any NADPH-dependent process.

Similar data were obtained when palmitoyl-CoA, *cis*-5-tetradecenoyl-CoA, *cis*-5-decenoyl-CoA, tetradecanoyl-CoA and *cis*-4-decenoyl-CoA were used as substrates. Of these substrates, the oxidation of *cis*-4-decenoic acid has been studied extensively and is mediated predominantly by a NADPH-dependent 2,4-dienoyl-CoA reductase pathway [8]. In the presence of malonate, the DNP-stimulated rates were lower for all saturated and unsaturated substrates studied. Therefore, the approach using oxygen-consumption rate under uncoupled and citric-acid-cycle-intermediate-depleted conditions did not provide unequivocal evidence about the requirement for NADPH in the oxidation of *cis*-5 or *cis*-4 fatty acids.

Oxidation of *cis*-5-dodecenoyl-CoA studied by metabolite measurement using a chromatographic method

When dodecanoic acid was used as substrate in rat liver mitochondria and studied by using a capillary column gas chromatograph, intermediates from the β -oxidation pathway could be detected as a result of incomplete oxidation [15]. The intermediates that are released generally include 3-hydroxy fatty acids,

trans-2 fatty acids and shorter-chain fatty acids from 1–3 cycles of β -oxidation. When *cis*-5-dodecenoic acid (100 nmol/ml) was studied (*n* = 3) under the same condition as described for dodecanoic acid, intermediates were also detected after 10 min of incubation. These intermediates included 3-hydroxydodecanoic acid (0.7 $\pm$ 0.6 nmol/ml), 3-hydroxydecanoic acid (1.0 $\pm$ 0.6 nmol/ml), 3-hydroxy-*cis*-5-dodecenoic acid (0.2 $\pm$ 0.3 nmol/ml) and *trans*-2,*trans*-4-dodecadienoic acid (1.5 $\pm$ 1.0 nmol/ml) (this intermediate was previously mis-identified as *trans*-2,*cis*-5-dodecadienoic acid in [2]). Dodecanoic acid (ranging from 0.05 to 1.5 nmol/ml) was also found; the mitochondria blank values for dodecanoic acid were less than 0.05 nmol/ml. Significantly lower accumulation of 3-hydroxydodecanoic and 3-hydroxydecanoic acids under identical study conditions as for dodecanoic acid was observed.

A more detailed study was performed with *cis*-5-dodecenoyl-CoA as substrate. In addition to the condition of ADP-stimulated oxidation, the studies were also carried out in the presence of DNP or rotenone (Table 2). The metabolic rates of *cis*-5-dodecenoyl-CoA as well as dodecanoyl-CoA were inhibited by 31 $\pm$ 20 % under DNP-uncoupled condition in these studies (*P* = 0.05). The ADP-stimulated rates were 3.6 $\pm$ 1.2 nmol/min per mg of protein (*n* = 5) for *cis*-5-dodecenoyl-CoA and 4.3 $\pm$ 1.1 (*n* = 4) for dodecanoyl-CoA. In the presence of rotenone, the stimulation of metabolic rates that was reported in the literature for polyunsaturated fatty acids [10] was not found. Instead, the metabolic rates, as determined from the disappearance of

Figure 1 Metabolites produced from the incubation of *cis*-5-dodecenoyl-CoA (60 μ M) with intact rat liver mitochondria (1 mg of protein/ml)

Top, control mitochondria; bottom, mitochondria plus rotenone. Metabolites are identified as: 1, *cis*-3-decenoate; 2, *trans*-3-decenoate; 3, decanoate; 4, 3-hydroxyoctanoate; 5, *trans*-2-decenoate; 6, *cis*-5-dodecenoate (starting substrate); 7, *cis*-3-dodecenoate; 8, *trans*-3-dodecenoate; 9, dodecanoate; 10, 3-hydroxydodecanoate; 11, *trans*-3,*cis*-5-dodecadienoate; 12, *trans*-2-dodecenoate; 13, *trans*-3,*trans*-5-dodecadienoate; 14, *trans*-2,*trans*-4-dodecadienoate; 15, 3-hydroxy-*cis*-5-dodecenoate; 16, 3-hydroxydodecanoate; I, internal standard (pentadecanoate). The sample was analysed as trimethylsilyl derivatives on a SPB-1 fused-silica capillary column (0.25 mm internal diam. $\times$ 25 m) gas chromatograph. The column temperature was from 60° to 250 °C, with an increase of 4 °C/min.

substrates, were inhibited slightly to 2.9 ± 0.8 nmol/min per mg ($n = 4$, $P = 0.002$) for *cis*-5-dodecenoyl-CoA and to 3.7 ± 0.8 nmol/min per mg ($n = 4$, $P = 0.05$) for dodecanoyl-CoA.

DNP did not change the metabolite profile of *cis*-5-dodecenoyl-CoA. The intermediates found in the incubation milieu consisted of *trans*-2,*trans*-4-dodecadienoic acid as well as *trans*-3,*cis*-5- and *trans*-3,*trans*-5-dodecadienoic acids. These metabolites were derived from the dehydrogenation and isomerization of *cis*-5-dodecenoic acid. The addition of rotenone changed the profile of *cis*-5-dodecenoic acid metabolites to include 3-hydroxydodecanoic acid, 3-hydroxydecanoic acid and 3-hydroxyoctanoic acid (Figure 1, Table 2). Under all conditions, dodecanoic acid was detected in the range of 0.3–2.5 nmol/ml. A similar relationship was also found in experiments using *cis*-5-tetradecenoyl-CoA and *cis*-5-decenoyl-CoA as substrates.

The metabolism of *cis*-4-decenoyl-CoA was studied for comparison. In the presence of ADP, the metabolites detected were *trans*-2,*cis*-4-decenoic and *trans*-2,*trans*-4-decenoic acids. The addition of rotenone inhibits the metabolic rate of *cis*-4-decenoyl-CoA to the same degree as that of *cis*-5 fatty acids, and additional metabolites (3-hydroxydecanoic acid and 3-hydroxyoctanoic acid) were detected.

Metabolism of [2,2'- 2 H $_2$]*cis*-5-dodecenoyl-CoA

When [2,2'- 2 H $_2$]*cis*-5-dodecenoyl-CoA (26% d_2) was incubated with rat liver mitochondria, the produced dodecanoate contained

Table 3 Reductase activities in rat liver mitochondria

Oxidation of NADPH, monitored as the decrease in the A_{340} , was taken as the measure for the activities of reductase using *cis*-5-dodecenoyl-CoA (30 μ M) or *trans*-2,*trans*-4-decadienoyl-CoA (30 μ M) as substrate. * Clofibrate (0.5%) in rat chow was fed to rats for 2 weeks. Data are expressed as means $\pm$ SD.

Substrate	Activity (nmol/min per mg of protein)	
	Control	Clofibrate*
c5MC12-CoA	4.0 ± 1.4 (4)	4.4 ± 1.6 (4)
t2t4MC10-CoA	14.7 ± 0.6 (5)	31.0 ± 0.6 (5)

9.9% and 9.3% d_2 in two experiments. The labelling pattern indicated that some dodecanoate was obtained from the direct reduction of the *cis*-5 double bond under the experimental condition.

Reductase activity

The enzyme activities were measured by the spectrophotometric method, using *cis*-5-dodecenoyl-CoA and *trans*-2,*trans*-4-decadienoyl-CoA as substrate for 5-enoyl-CoA reductase and 2,4-dienoyl-CoA reductase respectively. As shown in Table 3, a significant (about 2-fold) increase in the activity of 2,4-dienoyl-

CoA reductase was obtained; however, the activity of 5-enoyl-CoA reductase was not changed after clofibrate treatment.

DISCUSSION

Interpretation of the fatty acid metabolic pathway indirect data using measurement of oxygen-consumption rates is more complicated than the direct data from metabolite quantification by chromatographic method. Oxygen-consumption rates are dependent on the final products of fatty acid oxidation and other factors [20]. DNP disrupts NADPH production of energy-dependent transhydrogenases by uncoupling mitochondria [10]. Malonate further blocks the contribution of NADPH from isocitrate dehydrogenase. Under conditions of malonate and DNP, the production of NADPH is supposed to be decreased and NADPH-dependent processes are inhibited [10]. Even though the inhibition of *cis*-5-dodecenoyl-CoA oxidation under uncoupled conditions is consistent with the involvement of NADPH in oxidation of the *cis*-5 double bond, the similarly decreased rates from dodecanoyl-CoA, which is not dependent on NADPH for metabolism, renders this argument less conclusive. Furthermore, the stimulation of *cis*-5-dodecenoyl-CoA or *cis*-4-decenoyl-CoA oxidation by rotenone was not observed when these substrates were studied directly by chromatographic methods. The stimulation of oxidation of long-chain polyunsaturated fatty acid by rotenone was cited by Osmundsen et al. [10] as additional evidence for the operation of a NADPH-dependent pathway in these substrates. In their rationalization, rotenone increases the mitochondrial NADH/NAH⁺ ratio and, in turn, drives the increased production of NADPH by transhydrogenases. Although we do not exclude the possibility that the difference between our results and those reported in the literature is due to the substrates used (acyl-CoA plus carnitine versus acylcarnitine), it is more likely that the difference is due to artifacts from low oxygen-consumption rates with long-chain substrates. In their studies, unsaturated fatty acids did not show uniformly the trends expected from the inhibition or stimulation of NADPH production. Only substrates that show very low oxygen-consumption rates behaved in the direction predicted. Low oxygen-consumption rates are prone to artifacts of

measurement from high background rates induced by DNP or rotenone.

Another complication of the previous studies is the substrates used. Long-chain polyunsaturated fatty acids were used as substrates to compare with shorter-chain fatty acids in previous studies [10,11]. The oxidation of fatty acids is chain-length dependent [21]. Besides, the positional relationship of other double bonds in a polyunsaturated fatty acid molecule affects the oxidation rates even of substrates of the same chain length [22]. Therefore, the oxidation of a *cis*-4 or *cis*-5 double bond which can be reached only after β -oxidation cycles and which also contain double bonds in other positions can be too complicated to interpret. Linoleic acid, which contains an odd-numbered double bond, was shown not to depend on NADPH for its metabolism [10], despite that the NADPH-dependent 2,4-dienoyl-CoA pathway was first described for this compound [8].

When *cis*-5-dodecenoic acid was oxidized in intact rat liver mitochondria, 3-hydroxydodecanoic acid was formed among other metabolites. This metabolite can be produced only from a reduction pathway, whether a direct reduction [2] or after dehydrogenation and isomerization [3,4], as shown in Scheme 1. Besides the metabolite produced from the reduction of the double bond, other metabolites, such as *trans*-2,*trans*-4-dodecadienoic and isomeric 3,5-dodecadienoic, acids from the first cycle of oxidation were also found. The dienoic acids were not accumulated from prolonged incubation or the treatment with rotenone. This indicated that *trans*-2,*trans*-4- and $\Delta^3\Delta^5$ metabolites were probably metabolic intermediates instead of end products from alternate pathways. The metabolite profile obtained in this study is more consistent with the reduction pathway proposed by Schulz and colleagues [3,4]. Compared with the oxidation of dodecanoyl-CoA studied under identical conditions [15], the amount of 3-hydroxydodecanoate detected was significantly less in the metabolism of *cis*-5-dodecenoyl-CoA. This is consistent with the postulated pathway, since the production of 3-hydroxydodecanoate from *cis*-5-dodecenoyl-CoA took more steps than that produced from dodecanoyl-CoA. Any of these additional steps could be potentially rate-limiting.

Rotenone treatment increases the intramitochondrial NADH/NAD⁺ ratio, which inhibits the activity of 3-

Scheme 1 Postulated reduction pathways of *cis*-5-dodecenoyl-CoA

Path A is the major pathway. This pathway uses 2,4-dienoyl-CoA reductase to reduce *trans*-2,*trans*-4-dienoyl-CoA to *trans*-3-enoyl-CoA. Path B is the direct reduction of *cis*-5-enoyl-CoA to saturated acyl-CoA before further β -oxidation steps. The contribution of this pathway is expected to be minor, since the inhibition of ACDH abolished most of the production of 3-hydroxydodecanoate without an increased accumulation of dodecanoate. Abbreviations: R, C₅H₁₁; ACDH, acyl-CoA dehydrogenases; Iso I, $\Delta^3\Delta^5$ -enoyl-CoA isomerase; Iso II, $\Delta^{3,5}$, *trans*-2,*trans*-4-dienoyl-CoA isomerase; Red, 2,4-dienoyl-CoA reductase; EH, enoyl-CoA hydratase.

hydroxyacyl-CoA dehydrogenase and leads to the accumulation of 3-hydroxy fatty acids [15]. In this condition, the concentration of 3-hydroxydodecanoate increased as expected. However, the concentration of 3-hydroxy-*cis*-5-dodecenoate also increased. If the further metabolism of 3-hydroxy-*cis*-5-dodecenoyl-CoA by 3-hydroxyacyl-CoA dehydrogenase was not involved in the pathway of *cis*-5-dodecenoyl-CoA metabolism, as we suggested previously from studies with freeze-thawed mitochondria [2], then the treatment with rotenone would not be expected to have an effect on the concentration of 3-hydroxy-*cis*-5-dodecenoic acid. These data indicated that the conventional pathway for the oxidation of *cis*-5 fatty acids might be operating to some degree. In fact, using reconstituted enzymes, we have shown in previous studies that *cis*-5-enoyl-CoAs can be shortened by two carbon atoms to *cis*-3-enoyl-CoAs by the conventional pathway [23]. If we assume that the relative concentration of 3-hydroxy fatty acids accumulated in the incubation milieu is indicative of the flux through these pathways, then approx. 61% went through the reduction pathway, while the other 39% went through the conventional isomerase-mediated pathway. However, this estimate is based on the assumption that 3-hydroxyacyl-CoA and 3-hydroxy-*cis*-5-enoyl-CoA are equally good substrates for 3-hydroxyacyl-CoA dehydrogenases. Previous results from this laboratory have indicated that 3-hydroxy-*cis*-5-enoyl-CoAs were significantly poorer substrates than 3-hydroxyacyl-CoAs for 3-hydroxyacyl-CoA dehydrogenases [23]. In this scenario, the estimate from the concentration of 3-hydroxy fatty acids would be under-estimated for the reduction pathway.

Although the present data favour the existence of a reduction pathway as depicted in Path A of Scheme 1 for *cis*-5 unsaturated fatty acids, the possibility of direct reduction of the *cis*-5 double bond as a minor pathway cannot be ruled out completely. In the studies described in this investigation, the concentration of dodecanoic acid was significantly increased. Dodecanoate can be produced directly from the reduction of *cis*-5-dodecenoyl-CoA. Alternatively, dodecanoate can be produced from the reduction of *trans*-2-dodecenoyl-CoA produced in the metabolic pathway shown in Scheme 1 as suggested by Schulz and colleagues [3]. To distinguish dodecanoate produced from the two pathways, the oxidation of [2,2'-²H₂]*cis*-5-dodecenoyl-CoA was investigated. The rationale for this study design was that dodecanoate produced from the direct reduction would contain two ²H labels, like the original substrate, whereas those produced from the reduction of *trans*-2-dodecenoyl-CoA would contain only one ²H atom, since the other ²H would be lost by dehydrogenation. In these studies, some dodecanoate with two ²H labels was detected. Therefore, direct reduction of the *cis*-5 double bond is possible, but may represent a minor pathway (Path B in Scheme 1).

The enzyme activity, 4 nmol/min per mg of protein, for the reduction of 5-enoyl-CoA measured from freeze-thawed mitochondria was not inducible by treatment of rats with clofibrate. Besides a possible activity of 5-enoyl-CoA reductase, the total reductase activity measured spectrophotometrically could include

partly the activity of 2,4-dienoyl-CoA reductase and 2-enoyl-CoA reductase. The freeze-thawed mitochondria used for the measurement contained all the enzymes necessary for the conversion of *cis*-5-enoyl-CoA into 2,4-dienoyl-CoA and 2-enoyl-CoA before the reduction which utilized NADPH. On the other hand, the activity of 2,4-dienoyl-CoA reductase, which is required in the reduction pathway depicted in Scheme 1 Path A, was significantly higher and was inducible by clofibrate treatment.

In conclusion, the metabolism of both odd- and even-numbered double bonds in fatty acids requires a NADPH-dependent reduction step. The observation that heart cells have high activities of transhydrogenase and isocitrate dehydrogenase to produce NADPH, yet do not have any significant biosynthetic pathways that can use NADPH [24], probably can be explained by the reductive metabolic pathways of unsaturated fatty acids. Heart cells use fatty acids as their major metabolic fuel. The efficient operation of this machinery would require a high activity of the NADPH-producing environment.

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REFERENCES

- 1 Stoffel, W., Ditzer, R. and Caesar, H. (1964) Hoppe-Seyler's Z. Physiol. Chem. **339**, 167–181
- 2 Tserng, K.-Y. and Jin, S.-J. (1991) J. Biol. Chem. **266**, 11614–11620
- 3 Smeland, T. E., Nada, M., Cuevas, D. and Schulz, H. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 6673–6677
- 4 Luo, M. J., Smeland, T. E., Shoukry, K. and Schulz, H. (1994) J. Biol. Chem. **269**, 2384–2388
- 5 Dommes, V., Baumgart, C. and Kunau, W.-H. (1981) J. Biol. Chem. **256**, 8259–8262
- 6 Mitzugaki, M., Nishimaki, T., Yamamoto, H., Sagi, M. and Yamanaka, H. (1982) J. Biochem. (Tokyo) **92**, 2051–2054
- 7 Kunau, W.-H. and Dommes, P. (1978) Eur. J. Biochem. **91**, 533–544
- 8 Schulz, H. and Kunau, W.-H. (1987) Trends Biochem. Sci. **12**, 403–406
- 9 You, S.-Y., Cosloy, S. and Schulz, H. (1989) J. Biol. Chem. **264**, 16489–16495
- 10 Osmundsen, H., Cervenka, J. and Bremer, J. (1982) Biochem. J. **208**, 749–757
- 11 Hiltunen, J. K., Osmundsen, H. and Bremer, J. (1983) Biochim. Biophys. Acta **752**, 223–232
- 12 Jin, S.-J. and Tserng, K.-Y. (1990) Biochemistry **29**, 8540–8547
- 13 Goldman, P. and Vegelos, P. R. (1961) J. Biol. Chem. **236**, 2620–2623
- 14 Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–77
- 15 Jin, S.-J., Hoppel, C. L. and Tserng, K.-Y. (1992) J. Biol. Chem. **267**, 119–125
- 16 Gornall, A. G., Bardawill, G. J. and David, M. M. (1949) J. Biol. Chem. **177**, 751–766
- 17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
- 18 Tserng, K.-Y. and Kalhan, S. C. (1983) Am. J. Physiol. **245**, E308–E311
- 19 Kimura, C., Kondo, A., Koeda, N., Yamanaka, H. and Mizugaki, M. (1984) J. Biochem. (Tokyo) **96**, 1463–1469
- 20 Brady, L. J. and Hoppel, C. L. (1983) J. Nutr. **113**, 2129–2137
- 21 Bremer, J. and Norum, K. R. (1982) J. Lipid Res. **23**, 243–256
- 22 Lawson, L. D. and Holman, R. T. (1981) Biochim. Biophys. Acta **665**, 60–65
- 23 Tserng, K.-Y., Chen, L.-S. and Jin, S.-J. (1995) Biochem. J. **307**, 23–28
- 24 Hoek, J. B. and Rydstrom, J. (1988) Biochem. J. **254**, 1–10