Spiropentaneacetic Acid as a Specific Inhibitor of Medium-Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: To study the structure–activity relationship between pentanoic acid analogues and the inhibition of fatty acid oxidation, a number of 4-pentenoic and methylenecyclopropaneacetic acid derivatives were prepared. All compounds inhibited palmitoylcarnitine oxidation in rat liver mitochondria, with 50% inhibition occurring at a concentration between 6 and 100 μM. However, only methylenecyclopropaneacetic acid (MCPA) and spiropentaneacetic acid (SPA) showed in vivo inhibitory activity in rats as indicated by the occurrence of dicarboxylic aciduria. Rats treated with SPA excreted metabolites derived only from fatty acid oxidation whereas MCPA-treated rats also excreted metabolites derived from branch-chained amino acid and lysine metabolism. SPA is a specific inhibitor of fatty acid oxidation without affecting amino acid metabolism. The site of inhibition is medium-chain acyl-CoA dehydrogenase (MCAD). In contrast, MCPA inhibited both MCAD and short-chain acyl-CoA dehydrogenase with a stronger inhibition toward the latter. The inhibition of fatty acid oxidation by both inhibitors was partially reversible by glycine or *l*-carnitine. Since SPA does not form a ring-opened nucleophile such as that proposed for MCPA in the inhibition of FAD prosthetic group in acyl-CoA dehydrogenases, we propose that the irreversible inhibition by SPA occurs by a tight complex without forming a covalent bond to the isoalloxazine ring in FAD.

In mitochondria, the metabolism of fatty acids requires several enzymatic steps. The first step is the carnitine-dependent transport of fatty acids inside mitochondria. This step requires carnitine palmitoyltransferase (CPT). Once the fatty acyl group is inside mitochondria, it is regenerated as acyl-CoA. Four enzymatic steps of β-oxidation are needed to shorten acyl-CoA by two carbons in each cycle. These steps are acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase mediated reactions. Reduced activity in some of these enzymes from genetic defects or from inhibition by xenobiotic metabolites has been described in humans (Viancy-Liaud et al., 1987; Tanaka & Coates, 1990; Bjorge & Ballie, 1985; Tonsgard, 1989). Inhibitors of a specific enzyme are useful in the study of the metabolic consequence of such an inhibition. A number of inhibitors of fatty acid metabolism have been described (Schulz, 1987; El-Aleem & Schulz, 1987); 2-bromopalmitate and tetradecylglycidic acid are inhibitors of CPT, and a number of other compounds, such as 4-pentenoic, 4-bromocrotonic, and 2-bromooctanoic acids, act at the level of 3-ketoacyl-CoA thiolase.

A few fatty acid derivatives are known to inhibit medium-chain acyl-CoA dehydrogenase (MCAD)[1] activity (Freund et al., 1985; Shaw & Engel, 1985; Wenz et al., 1981, 1985; Zhou & Thorpe, 1989). However, an inhibitor that is effective in vivo and also specific for MCAD has not been described. In a study using synthetic methylenecyclopropaneacetic acid (MCPA), the active metabolite of hypoglycin, to study disordered fatty acid metabolism, we came across a synthetic byproduct which specifically inhibits only MCAD, and this inhibition can be demonstrated both in vivo and in vitro in a rat model. The structures of this synthetic byproduct [spiropentaneacetic acid (SPA)] and other related analogues are shown in Figure 1.

Materials and Methods

Synthesis of Spiropentaneacetic and Methylenecyclopropaneacetic Acids. Ethyl 3,4-pentadienoate (8.2 g), synthesized according to the procedure of Grandall and Tindell (1970), was added over 30 min to a preheated solution of zinc–copper couple (4.7 g), diiodomethane (19 g), and iodine (0.09 g) in 30 mL of ether (Ullman & Fanshawe, 1961). After refluxing for 15 h, the mixture was filtered and washed with saturated ammonium sulfate, saturated sodium bicarbonate, and water. After drying over sodium sulfate, the ether solution was concentrated. The product was a mixture of ethyl methylenecyclopropaneacetate (ethyl MCPA) and ethyl spiropentaneacetate in various proportions. The mixture was separated by high-performance liquid chromatograph with a semipreparative column [Alltech Econocil C_{18} column, 25 cm × 10 mm, 10 μm, methanol/water/acetonitrile (10:9:1), 8 mL/min, UV detection at 202 nm]. Ethyl spiropentaneacetate was eluted later and was completely resolved from ethyl MCPA. Gas chromatography of this product on an SPB-1 capillary column (12.5 m, temperature programmed from 80 to 250 °C at 4 °C/min) showed a symmetrical peak without detectable contamination of ethyl MCPA. The mass spectrum (70 eV, EI) with gas chromatographic inlet showed the expected fragmentation: *m/z* 154 (0.5, M^+), 126 (16, M – C_2H_4), 125 (8, M – C_2H_5), 112 (11), 111 (20), 108 (8, M – C_2H_5OH), 98 (17), 97 (12), 81 (100, M – $CO_2C_2H_5$), 80 (28),

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[1] Abbreviations: SPA, spiropentaneacetic acid; MCPA, methylenecyclopropaneacetic acid; 3,4-MP, 3,4-methylenepentanoic acid; 4,5-MP, 4,5-methylenepentanoic acid; SCAD, short-chain acyl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; EI, electron-impact induced ionization.

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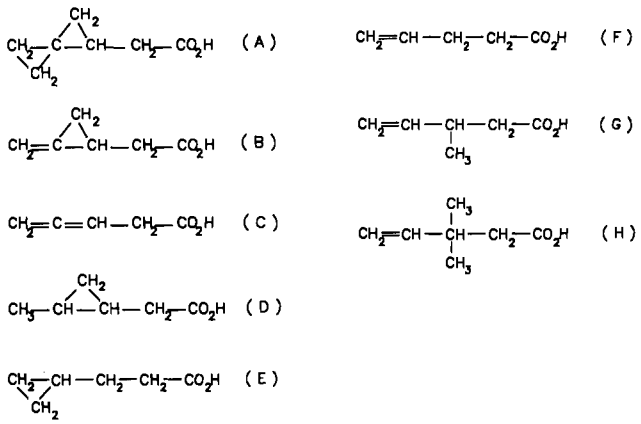

FIGURE 1: Chemical structures of fatty acid inhibitors related to methylenecyclopropaneacetic acid and 4-pentenoic acid: (A) spiropentaneacetic acid; (B) methylenecyclopropaneacetic acid; (C) 3,4-pentadienoic acid; (D) 3,4-methylenepentanoic acid; (E) 4,5-methylenepentanoic acid; (F) 4-pentenoic acid; (G) 3-methyl-4-pentenoic acid; (H) 3,3-dimethyl-4-pentenoic acid.

79 (59), 77 (7), 70 (10), 67 (18, M – $CH_2CO_2C_2H_5$), 55 (11), 53 (21). SPA as a sodium salt was obtained by alkaline hydrolysis of the ethyl ester with 1 equiv of sodium hydroxide in a minimum amount of 80% ethanol at 100 °C. After 5 min, the precipitated product was isolated by filtration and washed with ice-cold ethanol and ether. The NMR spectrum (200 MHz, in D_2O) showed multiplets centered at δ 2.10 (1 H) and 2.05 (1 H) due to CH_2CO; a single peak at δ 0.61 (4 H) assigned to the four terminal ring protons; a multiplet at δ 1.19 (1 H) due to the single proton on C-3; and two multiplets at δ 0.85 (1 H) and 0.40 (1 H) due to the other two ring protons (Ullman & Fanshawe, 1961).

Similarly, methylenecyclopropaneacetic acid (MCPA) was obtained as the sodium salt. The NMR spectrum (200 MHz, in D_2O) showed multiplets at δ 2.11 (2 H, CH_3COO), 1.59 (1 H), 0.77 (1 H), and 1.22 (1 H) and a doublet at δ 5.30 (2 H, CH_2=CC), consistent with the spectra obtained from similar compounds (Ullman & Fanshawe, 1961; Tanaka, 1972). Mass spectrum (70 eV, EI) of ethyl ester (% of base peak): *m/z* 140 (0.4, M^+), 112 (72, M – C_2H_5), 111 (13, M – C_2H_2), 98 (46), 97 (16), 95 (10), 84 (27), 83 (18), 67 (100, M – $CO_2C_2H_5$), 65 (29), 55 (14), 53 (12, M – $CH_2CO_2C_2H_5$). When injected into rats, this compound produced the same profile of dicarboxylic aciduria as that reported for hypoglycin (Tanaka, 1972; Hine & Tanaka, 1984).

Synthesis of Acyl-CoA's. The acyl-CoA derivatives of MCPA and SPA were synthesized by a modified method of Goldman and Vagelos (1961). The mixed anhydride was prepared by reacting the acid (0.1 mmol) with ethyl chloroformate (20 μL, 0.2 mmol) in the presence of triethylamine (28 μL, 0.2 mmol) in 2 mL of dry tetrahydrofuran (THF) for 10 min at 25 °C. This mixed anhydride with ethyl chloroformate was rapidly filtered through glass wool in a disposable Pasteur pipet. The filtrate was added dropwise over 10 min to a freshly prepared solution of lithium CoA (55 mg, 7 μmol) in 5 mL of water and THF (3:2 v/v) adjusted to pH 8 with sodium bicarbonate. When no free CoA was detectable using nitroprusside reagent (ca. 20 min), the mixture was adjusted to pH 3 and extracted with ether to remove unreacted free carboxylic acids. Organic solvents were removed by evaporation under vacuum. The dissolved ether was removed from the aqueous solution by bubbling nitrogen through; the solution was stored at −20 °C. The concentration of CoA derivative was determined by the method of Ellman (1959). The yields were between 71% and 85% (based on CoA). MCPA-CoA synthesized from MCPA generated by enzymatic deamination and decarboxylation of hypoglycin was reported to be extremely unstable by other investigators (Wenz et al., 1981; Billington et al., 1978). However, products synthesized in the presence procedure were as stable as the rest of the acyl-CoA derivatives. In an aqueous solution kept at −20 °C, no decrease in inhibitory activity toward acyl-CoA dehydrogenase was observed for at least 6 months.

High-Performance Liquid Chromatography. A Waters Associate (Milford, MA) HPLC system with two 510 pumps, a Model 680 automated gradient controller, and a Model 441 UV detector were used. For the analysis of synthetic acyl-CoA derivatives, an OD5 spherical C18 column (Burdick & Jackson, Muskegon, MI; 5 μm; 0.46 × 25 cm) was used. A gradient system of two solvents (A, 0.2 M sodium dihydrogen phosphate; and B, 20% acetonitrile in 0.2 M sodium dihydrogen phosphate) similar to that described by King and Reiss (1985) was used with a multiple step program from 0.6% acetonitrile for 2.5 min, which was then increased to 18% acetonitrile by the end of 10 min and kept at this composition for the rest of the analysis. In this system, *n*-butanoyl-CoA eluted at 16 min. The relative retentions of other acyl-CoA's to *n*-butanoyl-CoA were MCPA-CoA (1.08) and SPA-CoA (1.27).

In Vivo Study in Rats. Male Sprague-Dawley rats (200–250 g) which had been deprived of food for 17 h were injected intraperitoneally with a solution of MCPA and SPA in normal saline (30 mg/mL). The dose used was 80 mg/kg body eight. Fasting control rats were injected with an equal volume of normal saline. The rats were deprived of food for the duration of the experiments (48 h). Treated rats were placed in individual metabolic cages; urine specimens were collected every 24 h over toluene for the next 2 days. Samples were stored at −20 °C until analysis.

Rat urine (50 μL) mixed with internal standard (pentadecanoic acid, 20 μg in methanol) was extracted and converted to trimethylsilyl derivatives (Tserng et al., 1989). Identification of metabolites was achieved using a Hewlett-Packard 5985B gas chromatograph/mass spectrometer with a 15-m SPB-1 fused silica capillary column. Quantitative determination of the metabolites was performed with a dual capillary column gas chromatograph (Hewlett-Packard 5890A). Urinary creatinine concentration was determined by the standard colorimetric method.

Mitochondria Experiments. Overnight fasted female Holzmann rats were used for the isolation of liver mitochondria. The procedure for the isolation of mitochondria was essentially as described by Hoppel et al. (1979). Mitochondrial respiration rates were determined polarographically with a Clark-type oxygen electrode in a 0.5-mL chamber at 30 °C. Glutamate was used as a substrate to determine the quality of the mitochondria isolated. Only those preparations with a respiratory control ratio greater than 6 were used. Maximal oxidation rates were measured in the presence of excess ADP (2 mM). Fatty acid oxidation was determined using palmitoylcarnitine (8 μM) as substrate in the presence of malonate (5 mM) when acetoacetate was the product of fatty acid oxidation. The oxidation of other non-fatty acid substrates, i.e., pyruvate (10 mM) in the presence of malate (5 mM) and 2-ketoglutarate (10 mM) in the presence of malonate (5 mM), was also determined. Oxidative rates were expressed as nmol of O atoms/(min·mg of mitochondrial protein), determined by the biuret method.

Enzyme Assays. The mitochondria were incubated with normal saline (controls) or inhibitors. The enzymatic reaction

Table I: Selected Urinary Organic Acid Metabolites (mg/g of Creatinine) in Control Rats and in Rats Treated with Spiropentaneacetic (SPA) and Methylenecyclopropaneacetic (MCPA) Acids at 80 mg/kg Dose[a]

	controls (3)		SPA (3)		MCPA (3)	
metabolites	24 h	48 h	24 h	48 h	24 h	48 h
3-OH-isobutyric	7 ± 3	6 ± 2	4.8 ± 6.8	12 ± 2	570 ± 180	740 ± 393
2-Me-3-OH-butyric	ND	ND	ND	ND	55 ± 11	103 ± 42
glutaric	20 ± 13	27 ± 11	127 ± 68	15 ± 4	8559 ± 1015	11371 ± 914
isovalerylglycine	46 ± 14	28 ± 3	53 ± 3	83 ± 9	415 ± 13	677 ± 107
adipic	9 ± 4	7 ± 2	447 ± 48	28 ± 10	733 ± 112	1499 ± 26
suberic	13 ± 2	11 ± 2	181 ± 25	18 ± 5	250 ± 41	276 ± 28
sebacic	6 ± 6	5 ± 1	671 ± 49	15 ± 5	803 ± 100	142 ± 19
cis-4-octenedioic	47 ± 1	42 ± 14	535 ± 181	329 ± 163	350 ± 125	2045 ± 210
cis-4-decenedioic	24 ± 11	32 ± 14	4422 ± 1671	319 ± 28	7988 ± 1548	6739 ± 140
cis-4,7-decadienoic	ND	ND	441 ± 141	61 ± 28	947 ± 170	1384 ± 148
3-OH-decanedioic	12 ± 1	11 ± 3	123 ± 4	11 ± 4	111 ± 7	109 ± 19
glycine conjugate[b]	0	0	2224 ± 197	83 ± 29	648 ± 139	196 ± 31

[a] Only metabolites (mean ± SD) pertaining to the present discussion are listed. Urine samples were 24 -h collections after inhibitors. Inhibitors were administered intraperitoneally to rats after 17-h fasting. ND, not detected. [b] SPA-glycine and MCPA-glycine, respectively. These metabolites were determined as trimethylsilyl derivatives on a dual fused silica capillary column gas chromatograph (SPB-1 and SPB-35).

was terminated by freezing. Immediately before analysis, the thawed mitochondrial mixture was solubilized by the addition of 5% cholate and diluted with 0.1 M phosphate buffer, pH 7.2, to a final concentration of 1 mg of mitochondrial protein/mL and 1% cholate.

Acyl-CoA dehydrogenase activity was determined spectrophotometrically at 37 °C with 20 μg of mitochondrial protein in a 1-mL cuvette as described by Hoppel et al. (1979). Palmitoyl-CoA (50 μM), octanoyl-CoA (200 μM), and butanoyl-CoA (200 μM) were used as substrates for the measurement of long-chain, medium-chain, and short-chain acyl-CoA dehydrogenase activities, respectively. The activities are expressed as nmol of cytochrome *c* reduced/(min·mg of mitochondrial protein).

Inhibitory Activity of MCPA-CoA and SPA-CoA. Cholate-solubilized rat liver mitochondrial extract and partially purified beef liver mitochondrial extract (carnitine palmitoyltransferase activity has been removed from the soluble mitochondrial extract by Blue Sepharose chromatography) were used. The experiments were essentially identical to the assay of enzyme activity described above except that MCPA-CoA, SPA-CoA, or buffer (control) was added 3 min after mitochondrial soluble extract and 2 min before the addition of palmitoyl-CoA, octanoyl-CoA, or butanoyl-CoA.

Other Materials. 3,4-Pentadienoic acid was synthesized by the method of Grandall and Tindell (1970). 4,5-Methylenepentanoic and 3,4-methylenepentanoic acids were obtained by the reaction of methylene diiodide and zinc-copper couple with methyl 4-pentenoate and 3-pentenoate, respectively. 3-Methyl-4-pentenoic and 3,3-dimethyl-4-pentenoic acids were prepared according to the procedure of Jager and Gunther (1977). The sources of other reagents were as described before (Tserng et al., 1989; Hoppel et al., 1979).

Results

Urinary Organic Acid Profile in Rats Treated with Inhibitors. Rats treated with MCPA (80 mg/kg) had significantly elevated urinary excretion of metabolites derived from lysine (glutaric acid), leucine (isovalerylglycine), isoleucine (2-methyl-3-hydroxybutyric acid), and valine (3-hydroxyisobutyric acid) which resulted from the inhibition of glutaryl-CoA dehydrogenase, isovaleryl-CoA dehydrogenase, and 2-methylbutyryl-CoA dehydrogenase, respectively (Hine & Tanaka, 1984; Ikeda & Tanaka, 1990). In contrast, rats treated with the same dose of SPA did not have increased excretion of these metabolites (Table I). This indicates that the metabolism of lysine and branch-chained amino acids probably was not affected by SPA or its metabolite.

SPA-treated rats showed about the same degree of inhibition of fatty acid metabolism as MCPA (Table I). The dicarboxylic acids derived from saturated fatty acids (adipic, suberic, and sebacic) and unsaturated fatty acids (*cis*-4-octenedioic, *cis*-4-decenedioic, and *cis*-4,7-decadienedioic acids), as well as hydroxy acids (3-hydroxydecanedioic acid), were only slightly lower than those obtained from MCPA-treated rats. These effects occurred only during the first 24 h in SPA-treated rats, while those resulting from MCPA treatment lasted to 48 h. The detoxification of inhibitors by glycine conjugation is faster in SPA-treated rats than in MCPA-treated rats. This difference might explain the faster recovery from the excretion of abnormal urinary metabolites in SPA-treated rats. In the first 24 h after treatment, SPA-glycine was excreted about 4 times more than MCPA-glycine in rats (2224 ± 197 mg/g of creatinine vs 648 ± 139). From 24 to 48 h, the excretion of SPA-glycine was lower than that of MCPA-glycine (83 ± 29 vs 196 ± 31). At the same dose (80 mg/kg, ip), only one out of three rats treated with MCPA survived for more than 48 h, while all three rats treated with SPA survived and recovered completely.

Inhibitory Effect of SPA and Related Compounds on Fatty Acid Metabolism. Using palmitoylcarnitine as substrate and the measurement of oxygen consumption using a Clark type oxygen electrode, the inhibitory effect of SPA on oxygen consumption in intact rat liver mitochondria was dose related. The concentration that caused 50% inhibition (IC_{50}) was 20 μM. This inhibitory effect was the same whether exogenous ATP was added or not. Since the preincubation of mitochondria with the uncoupling agent, 2,4-dinitrophenol, removed the inhibitory effect of SPA on fatty acid oxidation, the active inhibitory species is likely to be SPA-CoA. Likewise, the IC_{50}'s of related compounds were determined: MCPA (100 μM), 4-pentenoic acid (7 μM), and 3,4-pentadienoic (12 μM), 3-methyl-4-pentenoic (13 μM), 3,3-dimethyl-4-pentenoic (60 μM), 4,5-methylenepentanoic (6 μM), and 3,4-methylenepentanoic (40 μM) acids. Despite potent inhibitory activities in vitro, all synthetic analogues with the exception of SPA and MCPA showed no change in the excretion of urinary metabolites when injected into fasting rats.

The inhibitory activities of SPA, MCPA, 4,5-methylenepentanoic (4,5-MP), and 3,4-methylenepentanoic (3,4-MP) acids on the oxidation of palmitoylcarnitine (PCN) and other non-fatty acid substrates (pyruvate + malate, 2-ketoglutarate + malonate) are listed in Table II. At the concentration that inhibited 50% oxidation of palmitoylcarnitine, the oxidation

Table II: Inhibition of Oxygen Consumption Using Various Substrates in Rat Liver Mitochondria after Incubation with Inhibitors[a]

	percentage activity of controls			
	MCPA (100 μM)	SPA (20 μM)	3,4-MP (40 μM)	4,5-MP (10 μM)
PCN	48 ± 7 (14)	51 ± 7 (19)	52 ± 9 (7)	54 ± 10 (8)
pyruvate	69 ± 19 (7)	68 ± 18 (7)	75 ± 15 (6)	64 ± 11 (6)
2-keto-glutarate	68 ± 17 (3)	70 ± 17 (3)	60 ± 5 (3)	57 ± 5 (3)

[a] Mean ± SD. The control activities [nmol of oxygen atoms/(min·mg of protein)] using various substrates were as follows: PCN (88 ± 18), pyruvate (46 ± 14), and 2-ketoglutarate (37 ± 6). The substrates were palmitoylcarnitine (PCN), 8 μM, + malonate (5 mM); pyruvate (10 mM) + malate (5 mM); and 2-ketoglutarate (10 mM) + malonate (5 mM). Oxygen consumption rates were measured with a Clark-type oxygen electrode maintained at 37 °C in the presence of excess ADP (2 mM). Numbers in parentheses are the number of experiments. Mitochondria were incubated with buffer (controls) or inhibitors for 2 min before the measurement of oxygen consumption rates were the addition of substrates.

Table III: Percentage Residual Activity of Acyl-CoA Dehydrogenases in Rat Liver Mitochondria Incubated with MCPA and SPA[a]

substrates	10 μM MCPA	20 μM SPA
palmitoyl-CoA	95 ± 3 (3)	111 ± 5 (3)
octanoyl-CoA	49 ± 11 (5)	64 ± 16 (3)
butanoyl-CoA	14 ± 7 (5)[b]	84 ± 19 (5)[b]

[a] Mean ± SD. The control activities [nmol/(min·mg of protein)] of various acyl-CoA dehrogenases were as follows: long chain (100 ± 7), medium chain (71 ± 3), and short chain (92 ± 1). Mitochondria, after incubating with either buffer (controls) or inhibitors for 2 min, were solubilized by cholate. The activity was measured spectrophotometrically at 37 °C of cytochrome *c* reduced. Numbers in parentheses are the number of experiments. [b] Signicantly different ($p < 0.05$) from the inhibition of octanoyl-CoA dehydrogenase (*t* test).

of pyruvate and 2-ketoglutarate was also inhibited but to a lesser degree than that of PCN. This inhibition of pyruvate and 2-ketoglutarate oxidation was the same for all four inhibitors including MCPA, which was shown by Billington et al. (1978) to have no inhibitory effect toward pyruvate and 2-ketoglutarate oxidation at a concentration that inhibited fatty acid oxidation.

As shown in Table III, when the mitochondria incubated with MCPA and SPA were solubilized and assayed for the activities of acyl-CoA dehydrogenases with palmitoyl-CoA, octanoyl-CoA, and butanoyl-CoA as substrates, the results show that MCPA was a more potent inhibitor of short-chain acyl-CoA dehydrogenase (SCAD) than toward medium-chain acyl-CoA dehydrogenase (MCAD). In contrast, SPA was a more specific inhibitor of MCAD than at SCAD. Both inhibitors did not show an inhibitory effect on long-chain acyl-CoA dehydrogenase. In contrast, 3,4-MP and 4,5-MP did not show inhibitory activity toward any of the three substrates in this assay. Since dilution (50-fold) of the incubation mixture was involved in the assay, this lack of inhibitory activity of 3,4-MP and 4,5-MP indicates that these inhibitors are reversible inhibitors. Despite a potent in vitro inhibitory activity in intact mitochondria, the dilution removed the inhibition. This is consistent with a lack of in vivo effect of these two compounds.

The irreversible nature of the inhibition by MCPA and SPA was further confirmed by the experiments with glycine or carnitine. As shown in Table IV, the inhibitory effect of MCPA and SPA toward fatty acid oxidation using palmitoylcarnitine as substrate in intact mitochondria was not completely removed by the subsequent incubation with glycine

Table IV: Effect of Glycine and *l*-Carnitine on the Inhibitory Activities of SPA and Related Compounds on Rat Liver Mitochondria Using Palmitoylcarnitine (8 μM) as Substrate[a]

conditions	MCPA (100 μM)	SPA (20 μM)	3,4-MP (40 μM)	4,5-MP (10 μM)
glycine(−)	47 ± 7 (4)	51 ± 7 (6)		
glycine(+)	60 ± 10 (4)[b]	69 ± 17 (6)[b]		
carnitine(−)	48 ± 5 (5)	53 ± 6 (7)	55 ± 2 (3)	62 ± 13 (3)
carnitine(+)	65 ± 22 (5)[b]	71 ± 10 (7)[b]	91 ± 6 (3)[b]	96 ± 18 (3)[b]

[a] Mean ± SD. Control activity was 76 ± 4 nmol of oxygen atoms/(min·mg of protein). Glycine (2 mM) or *l*-carnitine (2 mM) was added to mitochondria, which were preincubated with inhibitors, and further incubated for 2 min before the measurement of oxygen consumption rates with a Clark-type oxygen electrode using palmitoylcarnitine (8 μM) as substrate in the presence of malonate (5 mM) and excess ADP (2 mM). Numbers in parentheses are the number of experiments. [b] Significantly different ($p < 0.05$) from the values without glycine or carnitine (paired *t* test).

Table V: Percentage Residual Activities of Acyl-CoA Dehydrogenases in Soluble Extract of Rat Liver Mitochondria after Incubating with Synthetic MCPA-CoA and SPA-CoA

substrates	100 μM MCPA-CoA	20 μM SPA-CoA
octanoyl-CoA	26 ± 16 (7)	39 ± 11 (7)
butanoyl-CoA	10 ± 5 (5)	94 ± 3 (3)

[a] Mean ± SD. The control activities [nmol/(min·mg of protein)] were as follows: octanoyl-CoA (62 ± 17) and butanoyl-CoA (111 ± 6). Rat liver mitochondria were solubilized with cholate. The soluble extract was incubated with MCPA-CoA or SPA-CoA for 2 min before the measurement of activities of MCAD (using octanoyl-CoA as substrate) and SCAD (using butanoyl-CoA as substrate) spectrophotometrically.

or carnitine. In contrast, the inhibition observed for 3,4-MP and 4,5-MP was completely reversed by the addition of carnitine. These data indicate that the inhibition by MCPA and SPA was at least partially irreversible. Additional data to support the partial irreversibility of fatty acid oxidation inhibition by MCPA and SPA was obtained by the following experiments: solubilized mitochondrial enzyme extract was incubated with either MCPA-CoA or SPA-CoA to more than 50% inhibition of MCAD activity. After filtration through a Sephadex G-50 column, the enzyme extracts showed MCAD activities that were 17 ± 3% ($n = 4$) higher than their prefiltration values, indicating only partial removal of inhibition after the Sephadex column.

Since it is possible that the differential inhibition of MCPA and SPA toward acyl-CoA dehydrogenases could be due to the difference in the activation to their CoA ester, we synthesized the CoA esters of MCPA and SPA. The incubation of these CoA esters with the solubilized mitochondrial enzyme extract at the same concentration as that used for intact mitochondria experiments showed that the differential inhibition was not due to the activation process. The data shown in Table V indicate the same specific inhibitory activities. At the concentration of SPA-CoA that resulted in 39% of residual MCAD activity, the activity of SCAD was almost unaffected. In other experiments, this selectivity was also preserved when higher concentrations of SPA-CoA were used. SPA-CoA at 50, 100, and 200 μM concentrations resulted in 48 ± 18% ($n = 7$) residual activity of MCAD while the corresponding values were 93 ± 7% for SCAD. In contrast, MCPA-CoA at 200, 400, and 1000 μM inhibited MCAD to 36 ± 15% ($n = 6$) and SCAD to 7 ± 6% ($n = 7$) of original activities. The same specific inhibitory activity toward MCAD is also apparent when partially purified acyl-CoA dehydrogenases from beef liver mitochondria soluble extract was used as the source of enzymes (Table VI).

Table VI: Percentage Residual Activities of Acyl-CoA Dehydrogenase in Soluble Extract of Beef Liver Mitochondria after Incubating with MCPA-CoA and SPA-CoA[a]

substrates	100 μM MCPA-CoA	20 μM SPA-CoA
octanoyl-CoA	3 ± 6 (3)	57 ± 8 (4)
butanoyl-CoA	0, 0	95, 89

[a] Mean ± SD. The control activities [nmol/(min·mg of protein)] of acyl-CoA dehydrogenases in beef liver mitochondria soluble extracts were as follows: octanoyl-CoA (113 ± 27) and butanoyl-CoA (94, 95). Conditions were the asme as in Table V.

Discussion

In humans, a defect in or inhibition of fatty acid oxidation enzymes is generally expressed as dicarboxylic aciduria in fasting or hypoglycemic episodes, when mobilization of fatty acids increases. Medium-chain dicarboxylic acids (sebacic and *cis*-4-decenedioic acids) in urine are formed from the ω-oxidation of medium-chain monocarboxylic acids which may accumulate as a result of enzyme defects (Mortensen & Gregersen, 1981; Jin & Tserng, 1989). Shorter chain dicarboxylic acids (suberic, adipic, and *cis*-4-octenedioic acids) are derived from the β-oxidation of these medium-chain precursors. This β-oxidation takes place in mitochondria or peroxisomes (Kolvraa & Gregersen, 1986; Cerden et al. 1988; Vamecq et al., 1989; Suzuki et al., 1989). A number of inhibitors of fatty acid metabolism, in vitro, have been reported (Schulz, 1987). However, very few inhibitors have been tested for their in vivo inhibitory activities. In this investigation, the majority of the inhibitors of fatty acid metabolism in vitro failed to induce dicarboxylic aciduria in rats when injected intraperitoneally.

Methylenecyclopropaneacetic acid, the active metabolite of hypoglycin, is a potent inhibitor of acyl-CoA dehydrogenases, including short-chain and medium-chain acyl-CoA dehydrogenases in fatty acid metabolism and isovaleryl-CoA and 2-methylbutyryl-CoA dehydrogenases in branch-chained amino acids, as well as glutaryl-CoA dehydrogenase in lysine metabolism (Kean, 1976; Ikeda & Tanaka, 1990). The reduced analogues, 3,4-methylenepentanoic and 4,5-methylenepentanoic acids, despite a potent in vitro inhibitory activity, failed to exhibit any in vivo activity. However, the bicyclopropane analogue, i.e., spiropentaneacetic acid (SPA), showed a strong inhibition of fatty acid metabolism in vitro as well as in vivo. The in vivo difference between MCPA and SPA is that SPA elevated urinary excretion of metabolites derived only from inhibited fatty acid metabolism while glutaric acid, isovalerylglycine, and other metabolites derived from the inhibition of lysine and branch-chained amino acids were not affected. This suggests that SPA is a specific inhibitor of fatty acid metabolism. Of all the compounds studied, MCPA is the most potent inhibitor in vivo on fatty acid metabolism; however, its in vitro inhibitory activity is the weakest. Therefore, an in vitro assay for the potency of a fatty acid oxidation inhibitor does not predict the inhibitory activity in vivo.

MCPA is an inhibitor of both short-chain and medium-chain acyl-CoA dehydrogenases (Ikeda & Tanaka, 1990). Kean (1976) showed that MCPA was a specific inhibitor of SCAD only. However, other investigators reported that MCPA also inhibited MCAD (Wenz et al., 1981; Billington et al., 1978; Ikeda & Tanaka, 1990). Our data show that both SCAD and MCAD were inhibited by MCPA; however, the inhibition was more specific for SCAD. In contrast, SPA was a more specific inhibitor for MCAD. At a concentration that inhibited 50% or more of MCAD activity, SPA did not show significant inhibitory activity toward SCAD. The inhibitory activity toward other non-fatty acid substrates (pyruvate and 2-ketoglutarate) was significant, but it was less profound than for fatty acid substrate. MCPA and MCPA-CoA synthesized by enzymatic methods from hypoglycin were reported to have no inhibitory activity toward pyruvate and 2-ketoglutarate oxidation at a concentration that inhibited 50% of palmitoylcarnitine oxidation (Billington et al., 1978). The difference with the present data could be due to the purity and concentration of MCPA-CoA used. Hypoglycin used for the enzymatic production of MCPA was not obtained in pure form; the product always contained a significant amount of leucine (Billington & Sherratt, 1981). Despite the inhibitory activity toward pyruvate and 2-ketoglutarate, properties shared by other inactive (in vivo) fatty acids (3,4-MP and 4,5-MP), the inhibitory activity of SPA as well as MCPA was mostly irreversible. Since glycine and carnitine partially removed the inhibitory activity and so did Sephadex filtration, the in vitro inhibitory activity of SPA and MCPA was partly due to competitive inhibition. Their saturated analogues, 3,4-MP and 4,5-MP, are 100% reversible competitive inhibitors.

Either a methylenecyclopropyl or dicyclopropyl group is necessary for an irreversible inhibition of acyl-CoA dehydrogenases in fatty acid metabolism, both in vivo and in vitro. An irreversible inhibition appears to be required for the expression of in vivo inhibitory activity. Wenz et al. (1981) postulated a mechanism for the activation of MCPA-CoA to a reactive anion, which attachs irreversibly to the isoalloxazine ring of the FAD prothetic group of acyl-CoA dehydrogenases. While such a mechanism is feasible for MCPA-CoA, it is not possible for SPA-CoA since a similar ring opening is not favored for a spiropentane ring. Ullman and Fanshawe (1961) showed that SPA was stable to alkaline hydrolysis because no resonance stabilization can occur after α-proton abstraction and ring opening. Under the same condition, MCPA was ring-opened to 4-methyl-2,4-pentadienoic acid with the same mechanism proposed for the activation of MCPA to the reactive metabolite (Ullman & Fanshave, 1961; Wenz et al., 1981). An alternate mechanism to explain the irreversible inhibition could be that SPA-CoA, and possibly MCPA-CoA, simply binds very tightly to FAD without forming a covalent bond. Methylenecyclopropylformyl-CoA was shown recently to inhibit 2-methylbutyryl-CoA dehydrogenase without a significant activity on MCAD and SCAD (Melde el al., 1989, 1991). The inhibition is irreversible even though this compound cannot form a ring-opened, resonance-stabilized reactive intermediate as postulated for MCPA. It appears that a methylenecyclopropane or dicyclopropane ring structure is a necessary functional group for in vivo activity, while the addition or shortening of carbon number can shift the specificity of the inhibition toward different FAD-containing acyl-CoA dehydrogenases.

A tight complex between FAD and the inhibitor without forming a covalent-bonded adduct is also consistent with the partial reversibility of inhibition by glycine and carnitine. If the inactivation of acyl-CoA dehydrogenase by MCPA-CoA was through a covalent-bonded adduct as postulated by Wenz et al. (1981), then the reactivation of the enzymes should result in the formation of ring-opened metabolites of MCPA, such as hydroxylated methyl-substituted pentanoic acids. So far, ring-opened metabolites were not found in the urine of MCPA treated rats.

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Multibilayer Structure of Dimyristoylphosphatidylcholine Dihydrate As Determined by Energy Minimization†

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ABSTRACT: Complete energy minimization was carried out on the multibilayer crystal structure of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine dihydrate (DMPC·$2H_2O$), starting from the X-ray structure determination reported by Pearson and Pascher (1979) *Nature 281*, 499-501. The asymmetric unit contains two nonidentical DMPC molecules and four water molecules. Minimization removed the acyl chain disorder present in the X-ray structure and caused the carbon planes of the acyl chains to become mutually parallel. Two energy-minimized structures (structures I and II) were found which mainly differed in the hydrogen-bonding arrangement of the waters of hydration. In structure I as in the X-ray structure, one of the water molecules forms a hydrogen-bonded bridge between successive bilayers; but in structure II, all hydrogen bonds are satisfied on the same bilayer. Structure II corresponds to the global energy minimum and is also a suitable structure for single bilayers. The lattice constants and cell volume of the minimized structures are close to the experimental values. The electrostatic force between DMPC bilayers is attractive. The mean hydration energy of the water is −14.2 kcal/mol, which is 2.5 kcal/mol lower than the binding energy of ice.

Energy minimization has been carried out on multibilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine dihydrate (dimyristoylphosphatidylcholine dihydrate, DMPC·$2H_2O$),[1] using the X-ray crystal structure reported by Pearson and Pascher (1979) as the starting point.

Structural studies of phosphatidylcholines have been made by X-ray analysis, neutron diffraction, and NMR. The most

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[1] Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DLPE·HAc, 1,2-dilauroyl-DL-phosphatidylethanolamine–acetic acid; $DLPEM_2$, 2,3-dilauroyl-DL-glycero-1-phospho-*N*,*N*-dimethylethanolamine; DLG, 2,3-dilauroyl-D-glycerol; lysoPE, 3-palmitoyl-DL-glycero-1-phosphoethanolamine; DMPC1 and DMPC2, DMPC molecules 1 and 2 in the DMPC·$2H_2O$ crystal structure; W1, W2, W3, and W4, water molecules in the DMPC·$2H_2O$ crystal structure.

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