Induction of Medium Chain Dicarboxylic Aciduria by 2-Tetradecyloxiranecarboxylate (TDOC) in Rat

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Summary: Medium chain dicarboxylic aciduria has been induced in fasted rats by 2-tetradecyloxiranecarboxylate (TDOC), a known inhibitor of carnitine: palmitoyltransferase I. Administration to fasted rats in dosages of 5mg/kg or 30mg/kg of body weight each day caused suppression in the expected fasting-induced enhancement of 3-hydroxybutyric acid excretion and an increase in the excretion of palmitic acid in the first 24 hour period of fasting, implying an *in vivo* inhibition of the mitochondrial β -oxidation of long chain fatty acids. Along with these effects, excretions of two medium chain dicarboxylic acids—sebacic and suberic acids—were also induced in the fasted rats. A similar dicarboxylic aciduria was found in non-fasted rats given 30mg/kg of this inhibitor. Liver histology revealed microvesicular fatty change in the fasted, TDOC-loaded animals. The TDOC-induced medium chain dicarboxylic aciduria in rat is an effective model for correlation to impaired mitochondrial β -oxidation in human.

Key words: medium chain dicarboxylic aciduria — β -oxidation — fatty liver — 2-tetradecyloxiranecarboxylate (TDOC) — carnitine: palmitoyl transferase I — inborn error of metabolism — Reye's syndrome — rat

Introduction

Medium chain ($C_6 \sim C_{10}$) dicarboxylic aciduria has been reported in several inborn defects in fatty acid metabolism (Vianey-Liaud et al. 1987), and Reye's syndrome (Tonsgard, 1985), and is caused also by chemical substances both in human and in experimental animals (Sherratt and Veitch, 1984). The characteristics of such dicarboxylic acidurias are various and are dependent upon etiology.

Long chain fatty acids are mainly β -oxidized by an enzyme system occurring in the intramitochondrial compartment (Fig. 1). Long chain fatty acids may also be oxidized by the other system which is localized in the peroxisomal fraction. This peroxisomal β -oxidation system normally

serves to oxidize very long chain fatty acids (Lazarow and de Duve, 1976). It has been postulated that in situations where the mitochondrial β -oxidation mechanism is impaired, long chain fatty acids may be β -oxidized by the peroxisomal system, yielding medium chain monocarboxylic fatty acids. These medium chain fatty acids may further undergo ω -, or ω -1 oxidation, resulting in occurrence of medium chain dicarboxylic acids (Gregersen, 1984).

To investigate the various biochemical mechanisms of dicarboxylic acidurias encountered in human disease states, several animal models have been devised. Since an animal strain with spontaneous defects in the mitochondrial β -oxidation has not yet been discovered, either an inhibitor of the mitochondrial β -oxidation

Fig. 1. Pathways of fatty acid oxidation. Pathways of mitochondrial β -oxidation ($\downarrow$), peroxisomal β -oxidation ($\Downarrow$), and microsomal ω -oxidation ($\rightarrow$). C's: monocarboxylic acids, DC's: dicarboxylic acids, CPT-I: carnitine:palmitoyltransferase I, and TDOC: 2-tetradecyloxirane carboxylate (with an arrow indicating site of inhibitory effect).

has been administered, or the animal has been made riboflavin-deficient (Sherratt and Veitch, 1984), which decreases the β -oxidation activity (Hoppel et al. 1979). Sites of the metabolic block produced by most of these measures, however, have been not necessarily confined to the mitochondrial β -oxidation system, and hence the use of such treatment has made more

complicated the interpretation of the metabolic profile.

2-Tetradecyloxirane carboxylate (TDOC) is a long chain fatty acid analogue and has the chemical structure shown below.

This compound specifically interferes with mitochondrial β -oxidation of long chain fatty acids through inhibiting carnitine:palmitoyltransferase I (Tutwiler and Dellevigne, 1979). We can therefore expect that TDOC would induce production of dicarboxylic acids, when administered in vivo, but whether or not this actually occurs has not been properly investigated nor studied previously.

In this report, we present evidence that this compound produced medium chain dicarboxylic aciduria in rat, and discuss the observed characteristics of the chemical profile of the dicarboxylic aciduria.

Materials and Methods

Materials

Male Wistar strain rats each weighing 200-320 g were used. They were fed a standard stock chow ("CE-2", Clea Japan, Inc.), which contained 4.4 g fat and 1.4 mg of riboflavin per 100 g.

Sodium 2-tetradecyloxirane carboxylate dihydrate (McN 3802-21-98) was kindly supplied by McNeil Laboratories, Fort Washington. C₁₀ to C₂₆ saturated monocarboxylic fatty acids were purchased from Gasukuro Kogyo Inc., Tokyo. Pentadecanoic acid (PDA) was obtained from Aldrich Chemical Company, Inc. N,O-Bis (trimethylsilyl) trifluoro acetamide (BSTFA) was purchased from Pierce Chemical Co., Rockford.

Experimental protocol

All rats were fed ad libitum in the first

Fig. 2. Experimental protocol.

24 hour period, then they were divided into two groups; group 1 and group 2 (Fig. 2). Rats in group 1 were then fasted, and given 24 hour daily dosages of 0 mg/kg (group 1-a), 5 mg/kg (group 1-b), or 30 mg/kg (group 1-c) of TDOC for a further two 24 hour periods in six equal doses. Group 2 rats were fed ad libitum throughout the experiments. Both groups were equally loaded with TDOC at the same dosages as above for group 1, for a second and third 24 hour period. TDOC was administered intraperitoneally as a slurry in 0.5% (w/v) carboxymethylcellulose solution. Animals which received "0 mg/kg" of TDOC were injected with carboxymethylcellulose solution alone. Twenty-four hour urine was collected from all the animals in each 24 hour period.

At the end of each experiment, livers were immediately removed, weighed, and processed for electronmicroscopic observations.

Analysis of carboxylic acids

Carboxylic acids were extracted from urine as previously described (Tanaka et al. 1980), with a few modifications. A portion of urine sample containing 0.25 mg of creatinine was diluted with water to give 2.0 ml, 200 μ g of pentadecanoic acid added as an internal standard, and acidified with

6N-HCl. Carboxylic acids were then extracted into 2.0 ml aliquot of ethylacetate three times, followed by extraction into 2.0 ml of ethylether. The solvent layers were combined, dehydrated over anhydrous sodium sulfate, and dried under gentle nitrogen stream. A derivative of the extract was produced with 100 μ l of BSTFA at 60°C for 30 min, and this was then subjected to analyses by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS).

A Shimadzu GC-7A gas chromatograph with flame ionization detector was used. The columns used were fused silica wide bore capillary columns (Shimadzu CBP1-W25-100, 25m $\times$ 0.53 mm). Helium was used as the carrier gas at a flow rate of 20 ml/min. The oven temperature was programmed from 80°C to 250°C at a rate of 8°C/min, with an initial isothermal delay of 2 min. One microliter of the specimen derivative was injected. Compounds separated by GC were verified on a Hitachi M-80B gas chromatograph-mass spectrometer equipped with the same column as for GC, and connected on line to a Hitachi M-003 data reduction system.

The relative concentration of carboxylic acid was expressed as peak area of a given carboxylic acid related to that of 200 μ g PDA, multiplied by a factor of 100.

Observation of ultrastructure of hepatocyte

Liver tissue was prefixed in 2.5% glutaraldehyde buffered with 50 mM cacodylate, pH 7.4, postfixed in 1% osmium tetroxide, and embedded in Epon. Ultrathin sections (1 μ m in thickness) were doublestained with uranylacetate-lead citrate, and subjected to electron microscopy.

Statistical analyses

Difference among three or more mean values were first investigated by analysis of variance (ANOVA). When a difference was found to be significant at a level of $\alpha=0.05$, then a pair of mean values were

tested either by paired or unpaired Student's *t* test (where the difference in variance was not significant), otherwise they were compared by Aspin-Welch analysis. A difference was determined as significant at an α value of 0.05.

Results

Effects of TDOC in the fasted rats

In fasted rats which did not receive TDOC (group 1-a), excretion of 3-hydroxybutyric acid increased in the second, and further in the third, 24 hour period, as compared with values in the first 24 hour period, but without statistical significance (Table 1). Excretion of 3-hydroxybutyric acid was significantly less in rats which received 5mg/kg of TDOC each day (group 1-b), when compared with that in control rats (group 1-a) in the second ($p=0.009$) and in the third ($p=0.0046$) 24 hour periods, while rats which were given with 30mg/kg of TDOC each day (group 1-c) excreted significantly reduced amounts of 3-hydroxybutyric acid in the third 24 hour period ($p=0.0025$). A typical total ion current chromatogram is shown in Fig. 3. Concentrations of palmitic acid did not change significantly throughout the three 24 hour periods in rats which were not loaded with TDOC (group 1-a). Excretion of palmitic acid, however, increased significantly in the second 24 hour period ($p=0.0294$) in rats given 5mg/kg of TDOC each day (group 1-b), as compared with those for the same rats in the first 24 hour period. Excretion of palmitic acid by the group 1-b rats was significantly more than that in group 1-a rats ($p=0.0499$) in the second 24 hour period. Excretion of palmitic acid increased in the second and the third 24 hour periods in rats given 30 mg/kg each day of TDOC, with a significant difference from that in the first 24 hour period ($p=0.0076$).

The ratio of 3-hydroxybutyric acid to

palmitic acid was significantly depressed in the second 24 hour period in rats which were loaded with 5mg/kg (group 1-b: 0.06 ± 0.04 , $p=0.0015$) and 30mg/kg (group 1-c: 0.06 ± 0.04 , $p=0.0015$) of TDOC each day, as compared with the values in the control rats which were not given TDOC (group 1-a: 1.39 ± 0.38), indicating an impairment to ketogenesis from palmitic acid.

Excretions of both sebatic and suberic acids were significantly enhanced by each of 5mg/kg and 30mg/kg of TDOC each day in the second and in the third 24 hour periods, as compared with values of the two dicarboxylic acids in the first 24 hour period ($p<0.005$), barring concentration of sebatic acid which exhibited no significant increase in the third 24 hour period.

Since adipic acid was incompletely separated from malic acid under the analytical conditions employed, the peak areas of the two compounds were combined (Table 1). The following results are therefore given for the sum of adipic and malic acids. Excretions of adipic acid plus malic acid decreased in the second and in the third 24 hour periods ($p<0.0249$), in comparison with the values in the first 24 hour period, irrespective of whether or not the animals received TDOC.

Ratios of 3-hydroxybutyric acid to each of sebatic acid and suberic acid were significantly decreased, when each of the ratios for rats which received TDOC in daily dosages of 5mg/kg (group 1-b: 0.03 ± 0.03 for sebatic acid, $p=0.004$, and 0.04 ± 0.04 for suberic acid, $p=0.008$) or 30mg/kg (group 1-c: 0.04 ± 0.02 for sebatic acid, $p=0.004$, and 0.04 ± 0.03 for suberic acid, $p=0.008$) was compared with the figures for control rats (group 1-a: 0.46 ± 0.16 for sebatic acid, and 0.62 ± 0.14 for suberic acid, respectively).

Effect of feeding

In rats fed ad libitum (Table 2), analysis of variance demonstrated significant differences in the excretion of sebatic acid

TABLE 1
Effects of TDOC on concentrations of carboxylic acids in urine of fasted rats

Group	dose of TDOC mg/kg/24 hr	Carboxylic acids ¹⁾				
		3-hydroxy- butyric	Adipic + Malic	Suberic	Sebacic	Palmitic
first 24hr, fed						
1-a	0	1.59±0.96	11.86±5.13	2.27± 0.73	2.80± 2.75	0.74± 1.05
1-b	5	1.41±1.12	10.63±5.05	1.60± 0.94	2.05± 2.81	2.45± 2.40
1-c	30	0.55±0.84	12.94±3.66	2.40± 0.53	4.11± 3.76	5.86± 1.59
second 24hr, fasted						
1-a	0	1.95±0.79	1.58±1.90	3.14± 0.79	4.25± 0.53	1.46± 0.68
1-b	5	0.43±0.60	5.73±3.27	11.54± 4.41	11.70± 5.53	6.67± 4.13
1-c	30	1.27±0.76	3.82±2.50	32.60±16.82	39.83±21.50	19.84± 7.66
third 24hr, fasted						
1-a	0	3.05±0.98	0.51±1.14	3.18± 0.46	2.70± 1.38	1.43± 1.15
1-b	5	1.00±0.66	2.23±2.28	12.66± 4.22	11.08± 4.18	3.21± 1.32
1-c	30	0.87±0.54	4.26±4.35	39.60±28.80	32.56±33.91	14.25±12.02

¹⁾ Peak areas related to that of 200 μ g of pentadecanoic acid, multiplied by a factor of 100, per 0.25 mg creatinine (M±SD).

Fig. 3. A typical total ion current chromatogram of carboxylic acids in urine of a fasted rat loaded with 30 mg/kg of TDOC. Refer to following scan numbers for identification of trimethylsilyl (TMS) esters of the carboxylic acids: 134: 3-hydroxybutyric acid-mono-TMS, 286: adipic acid-di-TMS, 347: suberic acid-di-TMS, 415: sebacic acid-di-TMS, 433: pentadecanoic acid-mono-TMS (internal standard), and 463: palmitic acid-mono-TMS.

TABLE 2

Effect of TDOC on concentrations of carboxylic acids in urine of fed rats

Group	dose of TDOC mg/kg/24 hr	Carboxylic acids ^{b)}				
		3-hydroxy- butyric	Adipic + Malic	Suberic	Sebacic	Palmitic
first 24 hr, fed						
2-a	0	3.02±1.99	13.95±5.66	1.59± 1.52	0±0	0±0
2-b	5	1.55±1.67	8.64±6.15	1.80± 0.17	3.81± 5.71	1.76± 1.83
2-c	30	3.17±6.63	6.84±4.26	1.66± 1.16	0.72± 1.60	5.45± 2.52
second 24 hr, fed						
2-a	0	1.58±1.78	9.00±6.73	2.88± 2.98	2.22± 4.97	1.17± 2.44
2-b	5	3.72±5.32	9.51±5.32	1.45± 0.90	2.61± 2.40	2.14± 1.57
2-c	30	0.45±0.63	8.81±2.08	5.24± 5.79	7.81± 4.60	7.08± 3.50
third 24 hr, fed						
2-a	0	1.75±1.78	8.88±6.60	2.93± 2.06	0.72± 1.61	1.12± 2.49
2-b	5	2.20±1.44	10.76±6.55	1.47± 0.89	3.18± 4.51	0.98± 1.36
2-c	30	1.08±0.65	11.11±3.03	10.39±12.76	21.63±18.79	9.04±10.27

¹⁾ Peak areas related to that of 200 μ g of pentadecanoic acid, multiplied by a factor of 100, per 0.25 mg creatinine (M±SD).

Fig. 4. Ultrastructure of hepatocyte of a fasted rat loaded with 30 mg/kg of TDOC each day. Lengths of scales; 5 μ m for plate A, and 2 μ m for plate B.

in terms of dosage of TDOC and period of experiment, and in palmitic acid excretion with respect to dosage of TDOC only.

Excretion of sebatic acid in fed rats loaded with 30mg/kg of TDOC (group 2-c) was significantly increased in the second 24 hour period as compared with that in the first 24 hour period ($p=0.0069$).

Excretions of sebatic acid and suberic acid were significantly higher in the fasted rats than in the fed animals ($p<0.02$), each of which received 5mg/kg of TDOC, in the second and in the third 24 hour periods. When 30mg/kg of TDOC was given, the fasted animals excreted the acids in larger amounts than in the fed rats ($p<0.03$) in the second 24 hour period.

Ultrastructure of hepatocyte

In rats which were fasted, and loaded with either dosage of TDOC (groups 1-b and 1-c), an occurrence of fine fat droplets in the cytoplasm, not associated with any displacement of nuclei, was observed (Fig. 4A). Mitochondrial morphology was generally preserved intact. Some mitochondria appeared slightly swollen, while the electron density of the matrix and the configuration of the cristae remained unchanged (Fig. 4B). There were some increases in number of peroxisomes. Similar fine fat droplets were found in hepatocytes of rats which were fed and given 30mg/kg of TDOC (group 2-c), but not in those fed and given 5mg/kg of TDOC (group 2-b). The mitochondrial configuration was intact in both groups 2-a and 2-b of the animals. In rats which were fasted but not given the TDOC (group 1-a), fat droplets were not more than those encountered in fed rats without TDOC load, and the mitochondria were only minimally swollen.

Discussion

The present experiments demonstrate

that TDOC can induce medium chain dicarboxylic aciduria in the fasted rats. TDOC-induced dicarboxylic aciduria may be used to investigate biochemical mechanisms of particular dicarboxylic acidurias in human.

Long chain fatty acids are normally β -oxidized by the enzyme system which occurs intramitochondrially. When this process is impaired, long chain fatty acids may be alternatively β -oxidized by the peroxisomal system (Gregersen et al., 1984), the primary function of which is to oxidize very long chain fatty acids (Lazarow and de Duve, 1976). Medium chain monocarboxylic fatty acids, which are either produced as a result of chain shortening of long chain fatty acids, or endogeneously derived from adipose tissue, may further undergo ω -oxidation (Robbins, 1968) by a series of reactions, involving the microsomal cytochrome P-450 mediated monooxygenase system (Wada et al. 1968), to yield medium chain dicarboxylic acids (Mortensen and Gregersen, 1981).

Since it has been shown in vitro that TDOC block β -oxidation of long chain fatty acids through inhibiting specifically carnitine: palmitoyltransferase I (Tutwiler and Dellevigne, 1971), a key enzyme of mitochondrial β -oxidation, it is expected that mitochondrial β -oxidation of long chain fatty acids would be impaired in vivo by administration of this compound. The observations that the excretion of palmitic acid was more enhanced and that increased excretion of 3-hydroxybutyric acid in the second 24 hour period was suppressed by administration of TDOC in the fasted rats was consistent with the above mentioned presumption.

In this situation, medium chain dicarboxylic acids, i.e., sebatic, suberic and adipic acids, are very likely to be produced from medium chain monocarboxylic acids derived either from long chain fatty acids produced through β -oxidation via the peroxisomal pathway, or from endogeneous

medium chain monocarboxylic acids released from adipose tissue.

Dicarboxylic aciduria can occur associated with accelerated lipolysis, such as fasting or diabetes, both in human (Pettersen et al. 1972; Pettersen, 1974), and in experimental animals (Mortensen, 1981). In these conditions, however, those dicarboxylic acids excreted are mainly adipic acid, followed by less amounts of suberic acid and sebacic acid.

In contrast to these conditions, sebacic and suberic acids predominate when mitochondrial β -oxidation is functioning insufficiently (Gregersen et al. 1982; Vianey-Liaud et al. 1987). The observed profile of the dicarboxylic aciduria induced by TDOC was similar to that previously reported with mitochondrial β -oxidation defects. This profile of the TDOC-induced dicarboxylic aciduria further supports the previous suggestion that conditions with impaired mitochondrial fatty acid β -oxidation are associated with a type of dicarboxylic aciduria in which sebacic and suberic acids predominate.

The origin of the sebacic, suberic and adipic acids in the fasted, TDOC-loaded rats might be various and diverse. Possible biosynthetic pathways for these dicarboxylic acids include the following: 1. Long chain fatty acids are initially subjected to chain shortening via the peroxisomal β -oxidation system to give rise to those from C_{10} (decanoic) to C_8 (octanoic) monocarboxylic acids, and these acids further undergo ω -hydroxylation to yield C_{10} to C_8 dicarboxylic acids. 2. Long chain monocarboxylic acids are first ω -oxidized to become long chain dicarboxylic acids, then these carboxylic acids are β -oxidized to C_{10} to C_6 dicarboxylic acids, mainly in peroxisomes. And 3. C_6 (hexanoic) to C_{10} (dodecanoic) monocarboxylic acids released from adipose tissue are directly ω -oxidized to yield corresponding C_6 to C_{10} dicarboxylic acids. The last possibility, however, appears least likely, because contents of C_6

to C_{10} monocarboxylic acids constitute quite minor portions in the fatty acid composition of rat adipose tissue (Mortensen and Gregersen, 1981).

A unique characteristic of the TDOC-induced dicarboxylic aciduria is that sebacic and suberic acids predominated quantitatively over adipic acid. This phenomenon may be explicable by the following mechanism (Fig. 1). In the presence of the inhibition by TDOC of carnitine: palmitoyl transferase I, long chain fatty acids are β -oxidized alternatively via the peroxisomal pathway down largely to those from C_{12} (dodecanoic) to C_8 (octanoic) monocarboxylic acids. This peroxisomal β -oxidation of the fatty acids may be even more enhanced in the fasted rat, since it has been shown that fasting enhances the peroxisomal β -oxidation (Ishii et al. 1980). The three medium chain monocarboxylic acids thus formed, i.e., dodecanoic, decanoic and octanoic acids, further undergo microsomal ω -oxidation. ω -Hydroxylation is one of the regulatory steps in the ω -oxidation of such monocarboxylic acids. Affinity of the ω -hydroxylation system is highly specific toward each monocarboxylic acid, being highest toward C_{12} (dodecanoic) and C_{10} (decanoic) acids among C_6 to C_{12} monocarboxylic acids (Gregersen et al. 1983). In addition, it has been shown that the transport of sebacic acid into the mitochondria is the rate limiting step in the β -oxidation of this dicarboxylic acid, and that suberic acid is a poor substrate toward mitochondrial acyl-CoA dehydrogenases (Kølvra and Gregersen, 1986). These biochemical characteristics of subcellular organelles appear to make sebacic and suberic acids accumulate, when mitochondrial β -oxidation of long chain fatty acids is impaired by TDOC. It can be excluded that TDOC causes the accumulation of sebacic and suberic acids through inhibiting β -oxidation of medium chain mono-, or dicarboxylic acids, since β -oxidation of octanoate by isolated hepatocytes is not in-

hibited by TDOC (Tutwiler and Dellevigne, 1979).

This TDOC-induced dicarboxylic aciduria can simulate hepatic carnitine: palmitoyltransferase deficiency and systemic carnitine deficiency, but not medium chain- and long chain acyl-CoA dehydrogenase deficiencies, nor multiple acyl-CoA dehydrogenation defects in humans. Acylglycines, which are occasionally found in the latter three conditions, were all at levels below detection sensitivity in the rats in our present experiments. This is reasonable because the blocking sites in fatty acid oxidation are located in the mitochondrial matrix in these human diseases, while TDOC inhibits translocation of palmitoylmoieties into the mitochondrial matrix space.

TDOC could produce some of the biochemical and morphological features of Reye's syndrome. It has been postulated that generalized, though nonspecific, mitochondrial dysfunction constitutes a major component of the etiology of the syndrome, and dicarboxylic aciduria with a pattern similar to that of the TDOC-induced one has been found in patients with Reye's syndrome (Tonsgard, 1985). The morphological characteristics of the observed deposition of fat droplets in hepatocytes of the TDOC-treated rats were close to that seen in Reye's syndrome, i.e., small fat droplets evenly scattered over the cytoplasm of hepatocytes, without any nuclear shift to the periphery. Accordingly, TDOC may be useful to understand the nature of dicarboxylic aciduria and fatty changes which may be encountered in the above stated inborn error of fatty acid metabolism and in Reye's syndrome in human.

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