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Effect of 2-Methylcitrate on Citrate Metabolism: Implications for the Management of Patients with Propionic acidemia and Methylmalonic aciduria

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Extract

2-Methylcitrate was tested in vitro on enzymes which interact with citrate and isocitrate. It was found to inhibit citrate synthase, aconitase, the NAD^+- and $NADP^+$-linked isocitrate dehydrogenase. This inhibition was competitive in nature except in the case of aconitase, and the K_i for all the enzymes was in the range of 1.5–7.6 mM. Phosphofructokinase was also inhibited by 2-methylcitrate with 50% inhibition achieved at 1 mM. ATP-citrate lyase and acetyl-CoA carboxylase were not inhibited by this compound. 2-Methylcitrate was not a substrate for ATP-citrate lyase. Acetyl-CoA carboxylase was activated by 2-methylcitrate with a K_a of 2.8 mM. The apparent K_m (3.3 mM) for 2-methylcitrate for the mitochondrial citrate transporter was about 10-fold higher than the apparent K_m (0.26 mM) for citrate. The tricarboxylate carrier can also be inhibited by low concentrations (0.2 mM) of 2-methylcitrate when the concentration of citrate is close to the apparent K_m. Accumulation of 2-methylcitrate inside the mitochondrion, therefore, might lead to inhibition of enzymes in the citric acid cycle and thereby contribute to the ketogenesis and hypoglycemia seen under these conditions.

Speculation

Treatment of patients with propionic aciduria and methylmalonic aciduria with alkali therapy would be advantageous with respect to the acidemia but also would cause a more rapid exit of 2-methylcitrate from the mitochondrion. Alkalinization with sodium citrate might be even more beneficial if this citrate could enter the liver and allow more rapid removal of 2-methylcitrate and methylmalonate from liver mitochondria since increased cytosolic levels of these intermediates would facilitate more rapid diffusion to the extracellular space and eventual excretion in the urine. This therapy does not exclude the low protein diet and for the vitamin-responsive form of methylmalonic aciduria, B_{12} treatment.

In patients with propionic and methylmalonic acidemias, 2-methylcitrate has been found in the urine in substantial quantities (1). Propionyl-CoA accumulates in liver mitochondria as a result of a deficiency of one of the enzymes required for its conversion to succinyl-CoA. 2-Methylcitrate could be formed in the mitochondrion, for example, by condensation of propionyl-CoA with oxaloacetate, despite the fact that propionyl-CoA is a poor substrate for this enzyme (26). This study was undertaken to determine whether high concentrations of 2-methylcitrate could account for any of the metabolic abnormalities found in patients with these disorders. Therefore, the effect of 2-methylcitrate was studied on the enzymes and mitochondrial transport system known to be involved in the intermediary metabolism of citrate.

The movement of citrate from the mitochondrial to the cytosolic compartment on the tricarboxylate carrier subserves several important functions in the pathway of fatty acid synthesis. It is the major pathway for two-carbon unit flow from mitochondria to cytosol (17). In addition, citrate in the cytosol could contribute to regulation of acetyl-CoA carboxylation (9, 20). Operation of the mitochondrial citrate transporter (L-malate entry in exchange for mitochondrial citrate (4)) in conjunction with malate dehydrogenase and ATP-citrate lyase will also account for inward transport of cytosolic reducing power on NADH produced during the glycolytic sequence. Citrate is also an inhibitor of phosphofructokinase (5). More recently, an important regulatory role for mitochondrial citrate has been suggested in that this metabolite, in its free form, specifically inhibits pyruvate dehydrogenase phosphatase (5, 7). Regulation of the tricarboxylate carrier (7, 11, 12, 25) could be one

of the factors influencing the mitochondrial and cytosolic citrate levels.

MATERIALS AND METHODS

Livers were obtained from male Wistar rats (110–150 g (28)). The rats were allowed free access to food before being killed (1,000 hr). The methods employed for the isolation and loading of mitochondria with [14C]citrate as well as those for calculating the citrate exchanges were those of Robinson et al. (22–24). 1,2,3-Tricarboxybenzene (50 mM) was used for the "inhibitor stop" experiments. In order to study the kinetics of transport of 2-methylcitrate on the tricarboxylate carrier, this agent was used in the absence of [12C]citrate, whereas to demonstrate inhibition of this carrier, 2-methylcitrate was added in the presence of [12C]citrate.

Preparation and assay of enzymes from rat liver as well as liquid scintillation counting procedures were as described previously (5). Protein was measured by the technique of Lowry et al. (18) and citrate was determined by fluorometric analysis (19).

Enzymes, metabolic intermediates, and cofactors were obtained from Boehringer Mannheim (29). Rotenone, antimycin A, and dithiothreitol were from Sigma Chemical Co. (30). 1,2,3-Tricarboxybenzene was from K & K Laboratories (31). Sodium [14C]bicarbonate was from Amersham/Searle (32).

PREPARATION AND CRYSTALLIZATION OF 2-METHYLCITRIC ACID

The nuclear magnetic resonance spectrum was determined using a Varian A60 spectrometer and ^2H_2O as solvent. Sodium 2,2-dimethyl-2-silapentane-5 sulfonate ($\delta = 0$ ppm) was used as an internal reference.

The trisodium salt of 2-methylcitric acid was obtained according to the method of Habicht and Schneeberger (10). To obtain the free acid an aqueous solution of 1.0 g trisodium 2-methylcitrate was poured over a column of Dowex 50W-X8 (acid form) ion exchange resin. Evaporation of the aqueous eluent gave 2-methylcitric acid as an oil (10). The oil was allowed to stand for 2 days at room temperature during which time crystallization had commenced. The addition of a small amount (1 ml) of ether and subsequent scratching with a glass rod gave a crystalline mass.

Filtration gave 124 mg pure 2-methylcitric acid (melting point 145°–146°), obtained for the first time as a crystalline solid. The mother liquors were evaporated to give an oil, which on further standing for 24 hr partially crystallized to a solid mass. The residue was again taken up in a small volume of ether (1 ml) and a further crop of 84 mg 2-methylcitric acid (melting point 144°–146°) was obtained.

The nuclear magnetic resonance spectrum of 2-methylcitric acid showed a doublet at 1.25 ppm (3H, J = 7.2) (15) and a multiplet centered at 2.98 ppm (3H). The spectrum is entirely consistent with the assigned structure (16).

The mother liquors obtained from the crystallization of 2-methylcitric acid contained essentially pure 2-methylcitric acid as an oil. The nuclear magnetic resonance spectral evidence suggests that the crystalline 2-methylcitric acid is a pure diastereomeric compound, whereas the oil remaining from the crystallization is a mixture of the diastereomers of 2-methylcitric acid.

RESULTS

In all of the experiments, only the crystalline rather than the oil form a 2-methylcitrate had significant effects on the enzymes and transporter studied.

EFFECT OF 2-METHYLCITRATE ON ENZYMES INFLUENCED BY CITRATE (TABLE 1)

2-Methylcitrate is an inhibitor of citrate synthase, aconitase, the NAD+- and NADP+-linked isocitrate dehydrogenases. This inhibition is competitive in nature except in the case of aconitase. The

K_i for all of these enzymes is in the range of 1.6–7.6 mM. This compound, like citrate, is an inhibitor of phosphofructokinase; 50% inhibition was achieved with 1 mM 2-methylcitrate, whereas 3.9 mM citrate was required to achieve this degree of inhibition of phosphofructokinase under similar conditions (5). Pyruvate dehydrogenase was not inhibited by 2-methylcitrate in contrast to the effect of citrate (5). 2-Methylcitrate did not inhibit the cytosolic lipogenic enzymes, ATP-citrate lyase or acetyl-CoA carboxylase, and, in fact, 2-methylcitrate is a more potent activator of acetyl-CoA carboxylase than is citrate ($K_a \sim 6.7$ mM for citrate). 2-Methylcitrate was not a substrate for ATP-citrate lyase (Table 1).

EFFECT OF 2-METHYLCITRATE ON MITOCHONDRIAL CITRATE TRANSPORTER (TABLE 2)

To study the mitochondrial citrate transporter, mitochondria were isolated from rat liver and loaded with [14C]citrate (23). The [14C]citrate remained largely within the mitochondria until a counter transporting anion was added to the extramitochondrial space (23, 24). Although L-malate is the physiologic substrate for this transporter, citrate was chosen instead because it avoids the possibility of electrical or pH gradients which would be expected with the dicarboxylate anion, L-malate. In the experiments reported herein, the counter transporting anion was [12C]citrate or 2-methylcitrate. The apparent K_m for citrate for the tricarboxylate carrier of rat liver mitochondria from normal fed rats was 0.26 mM, whereas that for 2-methylcitrate was approximately 3.3 mM. However, the maximum velocity for both the substrates was similar (Fig. 1). The addition of 0.2 mM 2-methylcitrate inhibited the tricarboxylate carrier only at citrate concentrations which were close to the apparent K_m (0.2 mM), whereas this inhibition was no longer significant when the citrate concentration was 10-fold higher (Table 2).

DISCUSSION

2-Methylcitrate was observed in the urine in patients with propionic acidemia (1). The synthesis of 2-methylcitrate appears to be the consequence of propionyl-CoA accumulation and subse-

Table 1. *Effect of 2-methylcitrate on enzymes which interact with citrate or isocitrate*

Enzyme	Substrate	Inhibition K_i, mM
Aconitase	cis-Aconitate	4.0[1]
Citrate synthase	Acetyl-CoA	2.0
	Oxaloacetate	7.6
ATP-citrate lyase[2]	Citrate	No effect up to 5 mM
Isocitrate dehydrogenase, NADP+ linked (mitochondrial)	Isocitrate	1.8
Isocitrate dehydrogenase, NADP+ linked (extramitochondrial)	Isocitrate	1.6
Isocitrate dehydrogenase, NAD+ linked	Isocitrate	6.0
Acetyl-CoA carboxylase	Acetyl-CoA	0[3]
Phosphofructokinase	Fructose-6-PO4	50% inhibition by 1 mM
Pyruvate dehydrogenase	Pyruvate	No inhibition up to 4.5 mM

[1] All inhibitions were competitive except this one, which was noncompetitive.

[2] 2-Methylcitrate is not a substrate for this enzyme.

[3] Acetyl-CoA carboxylase was activated by 2-methylcitrate ($K_a = 2.8$ mM).

Table 2. *Effect of 2-methylcitrate on mitochondrial citrate transporter*[1]

Additions		Supernatant, dpm[2]
Citrate, mM	2-Methylcitrate, mM	
0.2	0	21,697
0	0.2	4,116
0.2	0.2	19,420
2.0	0	40,803
2.0	0.2	39,732

[1] Rat liver mitochondria (4.0 mg) were added to 1 ml of 20 mM Tris-HCl, 125 mM KCl buffer, pH 7.4, at 8° containing the additions as noted. After 1 min, 50 mM 1,2,3-tricarboxybenzene was added for the inhibitor stop technique. The mitochondria were separated by rapid centrifugation and a portion of the supernatant was prepared for counting as described previously. The total counts were 172,197 dpm and the supernatant fraction contained 38,746 dpm in the absence of additions. Results are reported as the mean of three values for a representative experiment. The total number of experiments was nine. The mean percentage of inhibition was 24.5% ± 5.5 when the citrate concentration was 0.2 mM and −2.1% ± 12.6 when the citrate was 2.0 mM.

[2] Disintegrations per min in supernatant after additions minus disintegrations per min in supernatant in the absence of tricarboxylate additions.
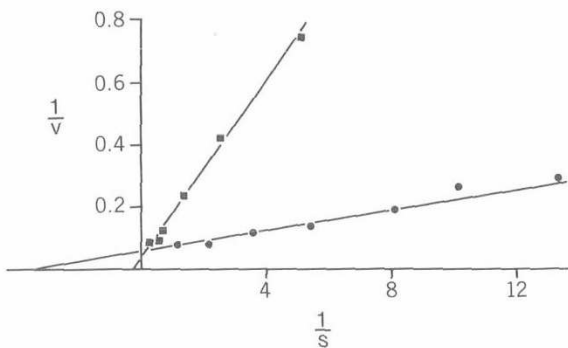

Fig. 1. Rate of exit of intramitochondrial [14C]citrate was studied as described in *Methods*. Mitochondria (3.7 mg and 4.8 mg, respectively) were incubated with [12C]citrate (●———●) or 2-methylcitrate (■———■) in the medium. The incubation was carried out at 8° and 1,2,3-tricarboxybenzene was added after 1 min. The apparent K_m for citrate was 260 μM and the V_{max} was 0.5 nmol/mg/min. The apparent K_m for 2-methylcitrate was 3.3 mM and the V_{max} 0.3 nmol/mg/min.

quent condensation with oxaloacetate catalyzed by citrate synthase. Although this enzyme is almost specific for acetyl-CoA (26), the enzyme from pig heart can use propionyl-CoA (26) as substrate but only at about 0.1% of the maximum rate observed with acetyl-CoA (26). 2-Methylcitrate might accumulate in mitochondria at levels high enough to affect the enzymes involved with citrate metabolism (Scheme 1). As discussed by Ando *et al.* (1), it is possible that another enzyme could be responsible for 2-methylcitrate synthesis.

It will be assumed for the remainder of the discussion that the crystalline form of 2-methylcitrate represents the physiologic species, as the oil form (which probably represents stereoisomers of 2-methylcitrate) had almost no significant effects on the enzymes and transporter involved in citrate metabolism. 2-Methylcitrate might accumulate in the mitochondrion as a result of the high K_m for 2-methylcitrate for the mitochondrial citrate transporter (Fig. 1). The metabolic consequence of accumulation of 2-methylcitrate within the mitochondrion can be hypothesized from the data in Table 1. Inhibition of the enzymes of the citric acid cycle, citrate synthase, aconitase, and isocitrate dehyrogenase,
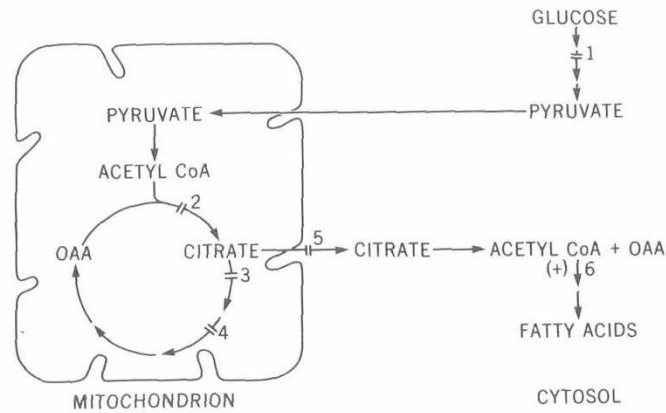
Scheme 1. Site of effects of 2-methylcitrate. *1:* phosphofructokinase; *2:* citrate synthase; *3:* aconitase; *4:* isocitrate dehydrogenase (NAD and NADP+ linked); *5:* mitochondrial citrate transporter; *6:* acetyl-CoA carboxylase (±, activation). *OAA:* oxaloacetic acid.

will lead to accumulation of acetyl-CoA. As a consequence, the acetyl-CoA/CoA—SH will increase and thereby result in increased ketone body synthesis (for review, see Reference 8). The accumulation of other acyl-CoA derivatives such as propionyl-CoA and methylmalonyl-CoA could further elevate the acetyl-CoA/CoA—SH by decreasing the free CoA—SH content and result in increased rates of ketogenesis.

As 2-methylcitrate accumulates in the mitochondrion the mitochondrial citrate transporter will be inhibited competitively. Citrate will thus rise in the mitochondria and fall in the cytosol. This could lead to a reduction in the rate of fatty acid synthesis and also lessen the citrate-induced inhibition of phosphofructokinase. Therefore, enhanced glycolysis could possibly contribute to the hypoglycemia reported in this condition (12, 20). Glycolysis would also be increased and gluconeogenesis inhibited if ATP were to fall as a result of inhibition of the citric acid cycle. The effects of 2-methylcitrate on the citrate transporter were similar to those reported for 2-ethylcitrate (22), namely that of competitive inhibition as well as a substrate of relatively low affinity.

As the mitochondrial 2-methylcitrate accumulates further, it will exit on the tricarboxylate carrier. 2-Methylcitrate will behave much like citrate in the cytosol, in that it is an activator of acetyl-CoA carboxylase and an inhibitor of phosphofructokinase (Table 1). In contrast to citrate, this compound is not a substrate for ATP-citrate lyase (Table 1). Therefore, the synthesis of odd chain length fatty acids, which is of importance in this condition (2, 3, 14, 15) would most likely result from exit of propionyl-CoA from the mitochondrion, probably as the carnitine derivative. 2-Methylcitrate could play an indirect role in the synthesis of odd chain length fatty acids by activating acetyl-CoA carboxylase (Table 1). Although not mentioned in the above discussion, the chelation of divalent cations by these carboxylic acids could also have profound metabolic effects.

SUMMARY

In summary, 2-methylcitrate accumulation inside the mitochondrion might lead to inhibition of the citric acid cycle and thereby contribute to the ketogenesis and hypoglycemia seen in these conditions. Treatment of such patients with alkali therapy would be advantageous with respect to the acidemia but also would cause a more rapid exit of 2-methylcitrate from the mitochondrion (6). Alkalinization with sodium citrate might be even more beneficial if this citrate could enter the liver and allow more rapid removal of 2-methylcitrate and methylmalonate from liver mitochondria (Fig. 1 and Reference 8). The increased cytosolic levels of these intermediates would facilitate more rapid diffusion to the extracellular space and eventual excretion in the urine.

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Brain   Niemann-Pick disease
fibroblasts   sphingomyelinase
liver

Sphingomyelinases in Human Tissues. II. Absence of a Specific Enzyme from Liver and Brain of Niemann-Pick Disease, Type C

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Extract

Sphingomyelinase was obtained in excellent yield from liver and brain by homogenization with 0.05 M citrate-phosphate buffer, pH 4.5, containing 0.25% Triton X-100 (v/v) followed by dialysis of the supernatant fluids against 1% glycine. Total recovery of enzyme was slightly less with tissue from Niemann-Pick disease compared with control tissue.