

The Effect of Malate on Propionate Mitochondrial Toxicity

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Propionic acidemia occasionally produces a toxic encephalopathy resembling Reye's syndrome, indicating disruption of mitochondrial metabolism. Liver mitochondria respiratory control ratios were reduced 46% by 5 mM propionate; inhibition correlated with matrix propionyl-CoA levels. L-Malate prevented the toxic effect of propionate and reduced the propionyl-CoA matrix concentration by 62%. The beneficial effect of L-malate is apparently due to stimulation of succinate efflux because the effect is blocked by benzylmalonate, an inhibitor of the dicarboxylate carrier. Matrix concentration of label from [1-¹⁴C]propionate was not affected by L-malate and/or benzylmalonate. L-Malate may be useful in the treatment of patients with propionic acidemia. © 1991 Academic Press, Inc.

Propionic acidemia is a rare inborn error of lipid and amino acid catabolism (1,2). Similar metabolic abnormalities occur in propionic acidemia and some other mitochondrial disorders (3,4). Propionic acid produces toxic acyl-CoA metabolites within mitochondria; their matrix levels correlate with reduction of the RCR (5).

Strategies for treating propionic acidemia have included protein restriction and carnitine therapy (2,6). Protein restriction removes a source of propionyl-CoA precursors; however, this approach is of limited benefit because endogenous sources account for a substantial proportion of propionyl-CoA precursors (7). Carnitine reduces the level of toxic propionyl-CoA by facilitating its conversion to nontoxic propionylcarnitine. However, patients still develop crises despite these therapies. We sought additional strategies which may be beneficial.

We hypothesized that L-malate may reduce propionyl-CoA levels by several mechanisms. First, citrate synthase utilizes propionyl-CoA and oxaloacetate (the product of malate oxidation) to synthesize methylcitrate. Second, malate stimulates efflux of propionyl-CoA catabolites, such as succinate or citrate, and may stimulate propionyl-CoA catabolism. L-Malate is translocated across the inner membrane on the tricarboxylate, dicarboxylate, and α -ketoglutarate carriers (8,9), compo-

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TABLE 1
Effect of Propionate, Malate, and Benzylmalonate on Oxidative Phosphorylation
in Liver Mitochondria

	RCR (<i>n</i> = 5)	State 3 (<i>n</i> = 5)
G	5.09 ± 0.34	82.4 ± 42.9
GP	2.36 ± 0.69 ^a	56.0 ± 46.0 ^b
GM	8.91 ± 1.23 ^c	172.9 ± 107.0 ^d
GPM	6.10 ± 0.84 ^e	118.8 ± 85.0
G+BM	1.70 ± 0.73	25.3 ± 32.3
GP+BM	1.14 ± 0.22 ^f	14.1 ± 15.1
GM+BM	2.23 ± 1.24	35.0 ± 43.0
GPM+BM	1.33 ± 0.35	21.6 ± 21.9

Note. G, glutamate + ADP; GP, glutamate + propionate + ADP; GM, glutamate + malate + ADP; GPM, glutamate + propionate + malate + ADP; G+BM, glutamate + benzylmalonate + ADP; GPM+BM, glutamate + propionate + malate + benzylmalonate + ADP. State 3 values are expressed as nanoatoms oxygen/min/mg protein, all summarized as means ± SD; *n* = number of rat mitochondrial preparations.

^a Significantly different from G, GM, GPM (*P* < 0.001, *P* < 0.001, *P* < 0.001) *t* test, unpaired samples.

^b Significantly different from G, GM, GPM (*P* < 0.05, *P* < 0.05, *P* < 0.05) paired *t* test.

^c Significantly different from G (*P* < 0.01) Mann-Whitney (Wilcoxin); different from GPM (*P* < 0.005) *t* test, unpaired samples.

^d Significantly different from G (*P* < 0.05) paired *t* test.

^e Significantly different from G (*P* < 0.05) *t* test, unpaired samples.

^f Significantly different from GM+BM (*P* < 0.05) Mann-Whitney (Wilcoxin).

nents of the malate-aspartate and malate-citrate shuttles (9). These carriers are antiport systems; citrate cycle counterions efflux as L-malate enters the matrix. Benzylmalonate is a specific inhibitor of the dicarboxylate carrier, but at higher concentrations also affects the phosphate/malate, α -ketoglutarate/malate, and citrate/malate transporters (8,10).

METHODS

We utilized previously described techniques for mitochondrial isolation, polarographic assays, protein measurement, and statistical analyses (5,11-14). The final mitochondrial pellet was resuspended to 20-40 mg protein/ml in the assay buffer. The assay buffer was 225 mM sucrose, 20 mM KCl, 5 mM MgCl₂, 20 mM EDTA in 10 mM potassium phosphate buffer, pH 7.4.

Mitochondria were incubated with glutamate alone or combinations of propionate, L-malate, and/or benzylmalonate (see Table 1 for coding). Concentrations in the final incubation mixture were: glutamate, 3.5 mM; L-malate, 3.5 mM; propionate, 5 mM; ADP, 2 mM; and benzylmalonate, 6 mM. Samples, prepared in triplicate, were incubated for 5 min, in the Thermolyne's Modular Dri-Bath at 30°C, then ADP was added to a concentration of 2 mM (to sustain the state 3 rate). Samples were withdrawn and placed into tubes containing 150 μ l of silicon oil (William F. Nye, Inc., New Bedford, MA; HI Phenyl Sil 125 DC) layered

TABLE 2
Matrix Volume and Matrix [$1\text{-}^{14}\text{C}$]Propionate Concentration

Sample	Matrix volume ($\mu\text{l}/\text{mg}$ protein) ($n = 5$)	Matrix [$1\text{-}^{14}\text{C}$]propionate ($\text{nm}/\mu\text{l}$) ($n = 5$)
G	1.22 ± 0.59	
GP	1.08 ± 0.60	6.58 ± 3.09
GM	1.17 ± 0.43	
GPM	1.24 ± 0.56	6.61 ± 2.09
G + BM	1.18 ± 0.65	
GP + BM	1.06 ± 0.72	9.58 ± 4.68
GM + BM	1.16 ± 0.64	
GPM + BM	1.20 ± 0.77	8.54 ± 4.46

Note. Values are expressed as means \pm SD; n = number of rat mitochondrial preparations.

over 14% (W/V) perchloric acid. Centrifugation for 1 min in an Eppendorf Model 5412 centrifuge drove the mitochondria and only a minimum volume of medium through the silicon oil into the perchloric acid, rapidly stopping the reaction in the sample. An aliquot of the perchloric acid extract was immediately neutralized to pH 5.4 to 6.0 using a mixture of 3 N KOH and 0.5 M K_2HPO_4 . The precipitate (KClO_4) was removed by centrifugation using Microfilterfuge tubes with 0.45- μm nylon-66 membrane filters (Rainin Instrument Co., Inc.) and the samples were stored at -30°C . Acyl-CoA compounds were quantitated using our modifications (5) of Corkey's method (15).

Mitochondrial matrix volumes were measured as described previously (5,12). Each sample contained 4 μCi of $^3\text{H}_2\text{O}$ and 4 μCi of [$\text{U}\text{-}^{14}\text{C}$]sucrose.

We used the unpaired t test for comparing groups. When variance was unequal (F test), the Mann-Whitney (Wilcoxin) statistic was utilized. With some variables the interexperiment variation was large and the *paired* t test was then used (16).

RESULTS

L-Malate stimulates glutamate state 3 rates (G and GM, Table 1). Propionate (GP) reduced mitochondrial RCR to 46% of control (G) (Table 1). Reduced RCR were largely due to inhibition of state 3. L-Malate prevented this propionate effect; RCR are similar in controls (G and GM) and samples with propionate and L-malate (GPM).

Benzylmalonate inhibits oxidation (G + BM). L-Malate (GPM + BM) does not appear to prevent the propionate effect (GP + BM) after incubation with BM.

Label accumulating from [$1\text{-}^{14}\text{C}$]propionate is similar in GP, GPM, GP + BM, and GPM + BM. Matrix volume was not affected by propionate, L-malate, and/or BM in liver mitochondria (Table 2).

Propionyl-CoA accumulated in the propionate sample (GP) and was reduced 62% by L-malate (GPM) (Table 3). Malate did not reduce propionyl-CoA levels when benzylmalonate was added (GP + BM and GPM + BM). Propionyl-CoA

TABLE 3
Mitochondrial Matrix Concentration of Acyl CoA Compounds

Sample	Propionyl-CoA	Acetyl-CoA	Free CoA	Succinyl-CoA	Glutathione-CoA	Propionyl-CoA/CoA
G	0.11 ± 0.08	0.14 ± 0.06	0.25 ± 0.07	0.16 ± 0.06	0.48 ± 0.28	0.47 ± 0.30
GP	1.23 ± 0.48 ^a	0.05 ± 0.05 ^b	0.13 ± 0.02 ^c	0.10 ± 0.12	0.15 ± 0.05 ^d	9.82 ± 5.55 ^e
GM	0.03 ± 0.01 ^f	0.10 ± 0.07 ^g	0.20 ± 0.07	0.16 ± 0.06	0.38 ± 0.25	0.20 ± 0.16
GPM	0.47 ± 0.12 ^h	0.06 ± 0.04 ⁱ	0.20 ± 0.06	0.11 ± 0.07	0.21 ± 0.06	2.68 ± 1.59 ^j
G+BM	0.03 ± 0.04 ^k	0.07 ± 0.05	0.19 ± 0.11	0.13 ± 0.12	0.44 ± 0.46	0.31 ± 0.38
GP+BM	1.04 ± 0.84 ^l	0.03 ± 0.03	0.24 ± 0.14	0.10 ± 0.11	0.26 ± 0.22	8.20 ± 12.2
GM+BM	0.05 ± 0.05 ^m	0.09 ± 0.08	0.36 ± 0.18	0.13 ± 0.14	0.40 ± 0.28	0.15 ± 0.14
GPM+BM	0.88 ± 0.61	0.08 ± 0.07	0.27 ± 0.22	0.14 ± 0.15	0.29 ± 0.10	3.57 ± 1.69

Note. Values are expressed as means ± SD; *n* = 5 rat mitochondrial preparations.

^a Significantly different from G, GM, GPM (*P* < 0.01, *P* < 0.01, *P* < 0.05) Mann-Whitney (Wilcoxin).

^b Significantly different from G (*P* < 0.05) *t* test, unpaired samples; different from GM (*P* < 0.01) *t* test, paired samples.

^c Significantly different from G (*P* < 0.01) Mann-Whitney (Wilcoxin); different from GM, GPM (*P* < 0.05, *P* < 0.05) paired *t* test.

^d Significantly different from G, GM (*P* < 0.01, *P* < 0.001) Mann-Whitney (Wilcoxin).

^e Significantly different from G, GM, GPM (*P* < 0.01, *P* < 0.01, *P* < 0.05) Mann-Whitney (Wilcoxin).

^f Significantly different from GP, GPM (*P* < 0.01, *P* < 0.01) Mann-Whitney (Wilcoxin).

^g Significantly different from GP (*P* < 0.01) *t* test, paired samples.

^h Significantly different from G (*P* < 0.001), *t* test, unpaired samples.

ⁱ Significantly different from G (*P* < 0.05) *t* test, unpaired samples.

^j Significantly different from G, GM (*P* < 0.01, *P* < 0.01) Mann-Whitney (Wilcoxin).

^k Significantly different from GP+BM, GPM+BM (*P* < 0.01, *P* < 0.01) Mann-Whitney (Wilcoxin).

^l Significantly different from GM+BM (*P* < 0.01) Mann-Whitney.

^m Significantly different from GPM+BM (*P* < 0.01) Mann-Whitney (Wilcoxin).

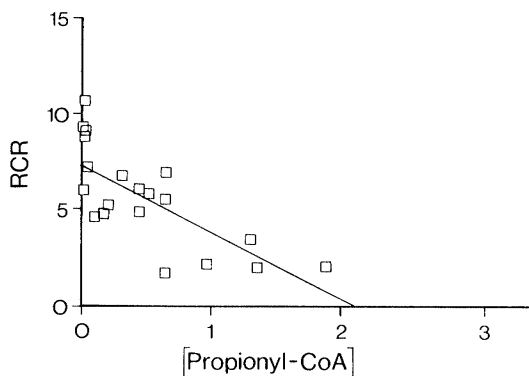


FIG. 1. The respiratory control ratio (RCR) is linearly related to the matrix concentration of propionyl-CoA (nm/ μ l); $r = -0.71$, $P < 0.001$, slope = -3.41 ± 0.78 ; $n = 20$.

levels correlated with the degree of inhibition of RCR (fig. 1) ($r = -0.71$, $P < 0.001$). Propionyl-CoA/free CoA ratios paralleled these changes (Table 3).

Free CoA and acetyl-CoA in GP are decreased compared to G (Table 3). Succinyl-CoA was not affected by L-malate or propionate. Glutathione-CoA is reduced by propionate.

DISCUSSION

Propionic acidemia occasionally produces a toxic encephalopathy resembling Reye's syndrome, indicating disruption of mitochondrial metabolism (1,2). Propionyl-CoA may be toxic because it inhibits several enzymes including citrate synthase (17,18), succinate:CoA ligase (GDP) (11), the pyruvate dehydrogenase complex (19), fatty acid oxidation (20), *N*-acetylglutamate synthetase (EC 2.3.1.1) (21), and short chain acyl-CoA dehydrogenase (22). Our previous data demonstrated that propionyl-CoA levels, modified by carnitine, correlated with the degree of inhibition of the RCR (5). Remarkably, in the current studies, L-malate prevented the propionate mitochondrial toxicity and also markedly reduced matrix propionyl-CoA concentrations (Table 3). Propionyl-CoA levels, in these studies modulated by L-malate, also correlated with the degree of inhibition of the RCR ($r = -0.7167$, $P < 0.001$) (Fig. 1).

We considered several mechanisms by which L-malate might decrease matrix propionyl-CoA:

- (1) Malate blocks transport of propionate into the matrix;
- (2) malate stimulates succinate, citrate, or α -ketoglutarate efflux;
- (3) malate inhibits propionyl-CoA production from propionate;
- (4) malate increases oxaloacetate and its condensation with propionyl CoA, which forms methylcitrate.

We disproved hypothesis 1 by direct measurement of matrix [14 C]propionate; its level was not affected by L-malate (Table 2). We do not have any direct evidence relating to hypotheses 3 or 4; testing these will require radiotracer flux studies (5) and quantitative measurements of methylcitrate. Propionate inhibition

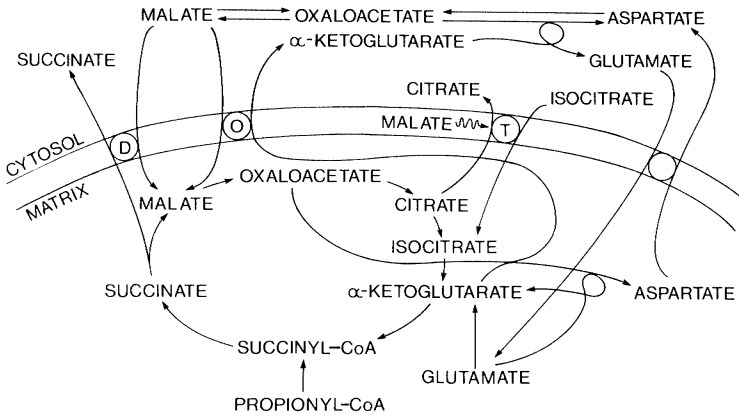


FIG. 2. Relationships between propionyl-CoA and L-malate metabolism. L-Malate is transported on the dicarboxylate (D) and α -ketoglutarate (O) carriers and stimulates the tricarboxylate (T) carriers. The glutamate-aspartate carrier participates in the aspartate-malate shuttle. L-Malate may lower propionyl-CoA levels by stimulating efflux of its catabolites, such as succinate.

of succinyl-CoA ligase (11) might impair malate generation and this could be overcome by exogenous malate (hypothesis 4). However, one might then expect a high succinyl-CoA level; this was not observed.

Hypothesis 2 seems tenable. L-Malate plays an important role in mitochondrial anion transport on the tricarboxylate (8-10,23-27), the α -ketoglutarate (8,24,28,29), and dicarboxylate (8-10,24,30) carriers. These carriers are components of the malate-aspartate (9,24) and malate-citrate shuttle (9) (Fig. 2). Benzylmalonate (BM) is an inhibitor of phosphate/malate exchange (8,10) and the dicarboxylate carrier (8,10); high concentrations (10 mM) also inhibit α -ketoglutarate and citrate transport (8,23). Benzylmalonate acts without penetrating the matrix (10,23). BM blocks the beneficial effect of L-malate on propionate inhibition (compare GP + BM with GPM + BM). L-Malate did not decrease matrix propionyl-CoA levels in the presence of BM (Table 3). Taken together, these data suggest that L-malate is increasing the efflux of succinate on the dicarboxylate carrier, pulling propionyl-CoA metabolism toward succinate. Halperin *et al.* (31) reported that methylmalonic acid inhibits L-malate transport across the mitochondrial membrane. A similar effect may occur with propionyl-CoA. This might be overcome by higher L-malate concentrations.

Acetyl-CoA, glutathione-CoA, and free CoA were decreased by propionate (GP; Table 3). Free CoA levels are restored by L-malate (GPM). However, acetyl-CoA and glutathione-CoA remain low in GPM. Thus, the low acetyl-CoA levels in GP do not account for the reduced RCR and state 3 rates. Reduced free CoA may contribute to the impaired oxidative metabolism.

In propionic acidemia patients, impaired growth and developmental retardation result from secondary vital nutrient deficiency. Roe and colleagues reported the successful treatment of propionic acidemia by hyperalimentation, prolonged oral L-carnitine administration (200 mg/kg/day for 90 days) and incremental changes

in the amino acid composition (7). Propionic acidemia patients excrete excessive acetylcarnitine, putatively due to high tissue acetyl-CoA. When L-malate was added to the hyperalimentation medium, much less acetylcarnitine was excreted, indicating a shift of acetyl-CoA, perhaps into citrate production. These data, together with our own, suggest that L-malate may be useful in the treatment of propionic acidemia patients.

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