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Effect of succinylacetone administration on brain heme metabolism and behavior in mice*†

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Tyrosinemia is a genetic disorder in which there is a defect in the tyrosine-degrading enzyme, fumarylacetoacetate hydrolase [1]. This causes the accumulation and excretion of the compound 4,6-dioxoheptanoic acid (succinylacetone). Succinylacetone is a potent inhibitor of delta-aminolevulinic acid (ALA) dehydrase, the second enzyme of the heme biosynthesis pathway [2–4] (Fig. 1).

Neurological dysfunction is one of the clinical manifestations of hereditary tyrosinemia. The cause of the neurological dysfunction has not been determined. In the present study, succinylacetone was administered to mice in order to determine if this compound can alter brain heme metabolism and affect behavioural changes.

Methods

Male Balb c/by mice weighing 27–32 g were used. The original progenitors were obtained from the Jackson Laboratory, Bar Harbor, ME.

Succinylacetone was obtained from the U.S. Biochemical Corp., Cleveland, OH. [1,4-^{14}C]Succinate (40–60 mCi/mmol) and delta[4-^{14}C]aminolevulinic acid hydrochloride (40–60 mCi/mmol) were purchased from New England Nuclear, Boston, MA. ALA and coproporphyrin I were bought from Porphyrin Products, Provo, UT, and the Sigma Chemical Co., St. Louis, MO.

Succinylacetone was dissolved in phosphate-buffered saline in a concentration of 5 mg/ml and injected intraperitoneally at a dosage of 40 mg/kg body wt every 8 hr. Treatment groups differed in the number of days of injection: 1, 2, 5, and 10 days. Each group contained four or five animals. A similar number of control animals received a volume of phosphate-buffered saline equivalent to that given to the succinylacetone-treated animals (0.2 to 0.3 ml).

The open field test, a standardized psychometric measurement of activity, was performed on all animals before initiation of treatment and on days 1, 2, 5, and 10 after treatment was begun. Activity was assessed prior to the injection of succinylacetone or phosphate-buffered saline (baseline), and 1 and 3 hr after the injection. The tests were performed in two custom-made arenas, each measuring 12 in. by 9 in. and subdivided into 3 by 3 in. squares. Observation was performed either visually or by a pair of orthogonally bisecting photoelectric eyes. Activity was measured as the number of boxes traversed or the number of times the light beams were interrupted during a 5-min span.

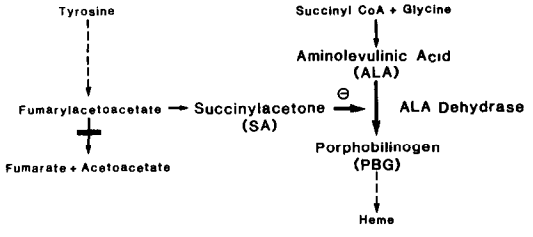

Fig. 1. Relationship between abnormal tyrosine degradation and heme biosynthesis in hereditary tyrosinemia. As a consequence of a defect in fumarylacetoacetate hydrolase, succinylacetone is formed. This compound is a potent inhibitor of ALA dehydrase, the second enzyme in the heme biosynthesis pathway.

Brain and liver tissue was obtained immediately after the animals had been killed by intracardiac puncture and perfused with chilled (4°), heparinized phosphate-buffered saline. Light microscopy of brain slices showed no contamination with erythrocytes after this perfusion. The fluorometric method of Morrison [5] was used to measure heme in brain homogenates.

The method of Burch and Siegel [6] was utilized to measure brain and liver ALA dehydrase activities. Both fresh and frozen (−70°) homogenates were used, as freezing did not alter the enzyme activity.

Brain ALA synthase activity was determined by the radiochemical method of Brooker *et al.* [7]. Only fresh homogenates were used in the assay. A succinyl-CoA generating system was not used in the assay since it did not enhance activity.

Protein was measured by the method of Lowry *et al.* [8], using bovine serum albumin as the standard. ALA was assayed by the method of Mauzerall and Granick [9] on 24-hr urine samples collected from animals that were housed in metabolic cages. Statistical analysis was by the unpaired Student's *t*-test and the Mann Whitney U test.

Results

Succinylacetone, given intraperitoneally in a dose of 40 mg/kg body wt, produced a parallel inhibition of mouse brain and liver ALA dehydrase actitivies. At 1 hr after injection, liver activity was 4% of control, and brain activity was 3% of control. When assayed at 8 hr after injection, liver and brain activities were 29% and 34% of control respectively.

When succinylacetone was given at 8-hr intervals, brain ALA dehydrase activity showed a sustained decrease over 10 days (Fig. 2). Activity was measured on tissue obtained 3 hr after injection. The mean activity during this period was 12% of control.

After 10 days of treatment, succinylacetone-treated animals excreted 353 ± 119 nmol (mean ± SEM, N = 5) of ALA in urine over a 24-hr period, while control animals excreted 9.3 ± 4.9 nmol.

Despite the inhibition of brain ALA dehydrase activity, the total brain heme level was not changed in animals treated with succinylacetone (Fig. 2). Brain ALA synthase activity was also not altered significantly. The activity was 25 ± 3 pmol/mg protein/hr (mean ± SEM, N = 5) after 10 days of succinylacetone treatment, compared to 35 ± 11 in controls.

Neither the acutely nor the chronically treated animals exhibited different behavior from control animals (Table 1).

* Portions of this study were published in abstract form in *Clin. Res.* **31**, 507A (1983) and in *Gastroenterology* **86**, 1325 (1984).

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Table 1. Open field activity of succinylacetone-treated mice*

Days after treatment	Baseline		One hour after injection		Three hours after injection	
	Succinylacetone	Control	Succinylacetone	Control	Succinylacetone	Control
Day 1 (N = 17)	81.4 ± 9.1	75.8 ± 6.2	65.0 ± 12.6	55.0 ± 8.1	39.4 ± 7.4	44.8 ± 8.1
Day 10 (N = 13)	60.1 ± 2.4	63.4 ± 2.7			34.0 ± 5.8	41.1 ± 4.5

* Each value given for open field activity is the mean ± SEM for the number of boxes traversed in 5 min. There was no significant difference between the succinylacetone-treated animals and the control animals.

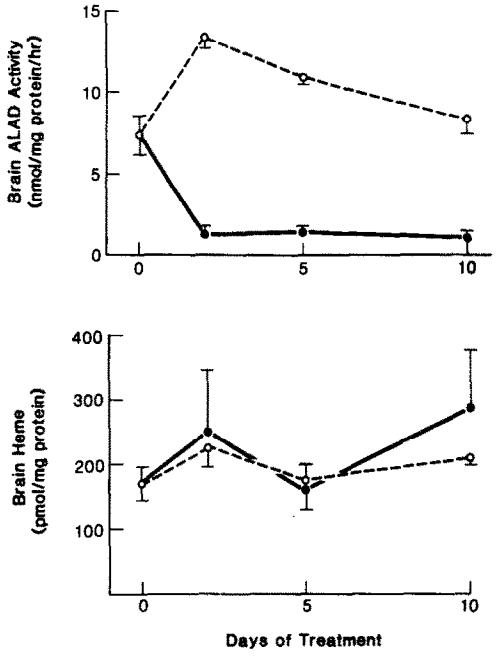

Fig. 2. Effect on brain ALA dehydrase (ALAD) activity and brain heme in animals given succinylacetone by intraperitoneal injection at 8-hr intervals (●), compared to animals given phosphate-buffered saline (○). Each data point is the mean ± SEM for four or five animals. Brain ALAD activity differed significantly at each time period ($P < 0.01$), whereas brain heme levels were not significantly different.

Discussion

In this study, succinylacetone crossed the blood–brain barrier and inhibited the heme biosynthesis enzyme ALA dehydrase when given intraperitoneally to mice. Nevertheless, the level of brain heme was not affected even by 10 days of treatment with succinylacetone. Brain heme exists predominantly in the form of mitochondrial cytochromes, which have an estimated half-life of 125–132 hr [10, 11]. There may also exist a smaller, rapidly turning over pool [11].

A possible reason for the failure of inhibition of brain ALA dehydrase to alter the brain heme level is the following. Under normal circumstances the amount of ALA dehydrase in tissue appears to be in great excess of that required for heme biosynthesis [2, 12]. In addition, the usual tissue concentration of ALA is probably well below the K_m for the enzyme [13]. Thus, when ALA dehydrase is partially inhibited, the residual enzyme activity may remain sufficient to provide adequate synthesis of porphobilinogen and maintain the brain heme level, particularly if the concentration of ALA increases. Since brain tissue levels of ALA were not measured in this study, it is uncertain whether succinylacetone treatment caused a significant increase in brain ALA content, however.

In liver tissue, heme biosynthesis is controlled at the level of ALA synthase. The enzyme is inducible and is under negative feedback control by heme. It is unknown whether brain ALA synthase is also inducible and regulated by heme [10, 11, 14, 15]. Our study provides no further information regarding this possibility, since neither the brain heme level nor ALA synthase activity appeared to be altered.

Behavior, as assessed by the open field test, was also not affected by succinylacetone treatment. It is of interest that behavior was unchanged despite a marked increase in excretion of ALA. It has been postulated that ALA acts as a neurotoxin due to its structural similarity to gamma-aminobutyric acid, the central nervous system inhibitory neurotransmitter [16].

In summary, following the intraperitoneal administration of succinylacetone to mice, brain ALA dehydrase activity was inhibited significantly, but this did not alter the brain heme or cause behavioral changes. Although inhibition of brain ALA dehydrase by itself may not cause neurological dysfunction, it may increase the susceptibility to other factors. This appears to be the situation in the inducible porphyrias, where enzyme defects in heme biosynthesis are necessary, but by themselves not sufficient, to cause neurological abnormalities. As a consequence, the succinylacetone-treated animal may still prove to be a useful model for studying the relationship between heme metabolism and neurological function.

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Differential effects of chymotrypsin on magnesium, sodium, and guanine nucleotide regulation of α_2-adrenoreceptors of human platelets

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The membrane-bound receptor–adenylate cyclase complex consists of at least three macromolecules, namely the receptors [R] located at the external surface of the plasma membrane, the catalytic unit of the enzyme [C] anchored in the inner surface of the plasma membrane, and the regulatory proteins [G] acting as couplers between the receptors and the catalytic unit of the enzyme [1]. The receptor–adenylate cyclase complex in human platelets where activation of α_2-adrenorcceptors leads to inhibition of adenylate cyclase is regulated by divalent [Mg^{2+}] and monovalent [Na^+] cations and by guanine nucleotide [GTP] [2]. We recently reported that pretreatment of human platelet membranes with trypsin eliminated the Mg^{2+} and GTP but not the Na^+ effect [3] and suggested that two distinct proteins are involved in Na^+ and GTP binding. In the present study, we investigated the effects of chymotrypsin on Mg^{2+}, GTP, and Na^+ regulation of the α_2-adrenoreceptors.

Materials and methods

The following chemicals and enzymes were purchased: chymotrypsin (bovine pancreas), trypsin inhibitor (soybean), 5′-guanylyllimidodiphosphate (Sigma Chemical Co.) and [*O-methyl*-^{3}H]yohimbine (90 Ci/mmol; Amersham).

Platelet membranes were prepared from platelet-rich plasma by differential centrifugation as described by Periyasamy and Somani [4].

Binding of [^{3}H]yohimbine to platelet membranes was carried out as previously described [4]. An 8nM concentration of [^{3}H]yohimbine was used in all experiments to measure total binding except in saturation binding experiments where concentrations of [^{3}H]yohimbine ranging from 0.5 to 20 nM were used. Nonspecific binding was measured in the presence of 10 μM unlabeled yohimbine, and specific binding was calculated by subtracting nonspecific from total binding.

Platelet membranes were treated with chymotrypsin as follows. Membranes were incubated with or without chymotrypsin [0.5 mg of enzyme · (mg membrane protein) · ml^{-1}] in a buffer containing 25 mM Tris–HCl + 0.5 mM EDTA (pH 7.5) at 35° for 3 min. Proteolytic activity of chymotrypsin was terminated (3 min later) by adding 2 parts of soybean trypsin inhibitor to 1 part of chymotrypsin by weight. The chymotrypsin-treated membranes were washed twice by repeated centrifugation at 40,000 *g* for 30 min. An aliquot of the washed membrane was used for binding assay.

Results

Effect of chymotrypsin on [^{3}H]*yohimbine binding.* Specific binding data generated from the saturation binding studies were transformed into a Scatchard plot, and the data were analyzed with computer-assisted linear regression analysis. As shown in Fig. 1, chymotrypsin reduced the number of binding sites from 302 ± 25 to 125 ± 20 fmol/mg protein without significantly affecting the affinity [K_d] of the receptors for [^{3}H]yohimbine. It also did not modify the affinity of the receptors for other antagonists such as phentolamine since the IC_{50} of phentolamine (82.0 ± 8.0 nM) was similar in control and treated membranes. However, the affinity of the receptors for the agonist was reduced, the IC_{50} for *l*-epinephrine being 950 ± 100 and 2300 ± 130 nM in control and treated membranes respectively.

Effect of chymotrypsin on the regulation of α_2-adrenoreceptors–agonist interactions by Mg^{2+}, GTP, and Na^+. Figure 2A shows that the epinephrine-displacement curve was shifted to the right by 2-fold in treated membranes as compared to control. Mg^{2+} increased the affinity of the receptors to epinephrine as shown by a shift in the displacement curve to the left; however, the ability of Mg^{2+} to increase the affinity of the receptors for epinephrine was consistently 2-fold less in treated membranes as compared to control, indicating that part of the Mg^{2+} effect was destroyed by chymotrypsin. GPP[NH]p reduced the affinity of the receptors for the agonist in control membranes but this effect of GPP[NH]p was lost in chymotrypsin-treated membranes (Fig. 2A). These results suggest that chymotrypsin inactivated the GTP binding component of the receptor–adenylate cyclase complex.

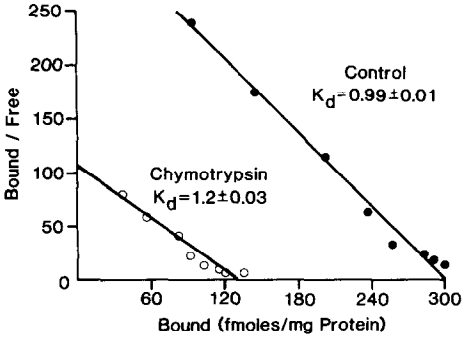

Fig. 1. Equilibrium binding data of specific [^{3}H]yohimbine binding to human platelet membranes pretreated with or without chymotrypsin. The data were converted into a Scatchard plot and were fitted by straight lines using linear regression analysis [μ = 0.97]. The data are the mean of three experiments conducted in duplicate.