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Effects of succinylacetone on methyl α-D-glucoside uptake by the rat renal tubule

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Succinylacetone, a catabolic end-product of tyrosine, is excreted in large quantities in urine from individuals with hereditary tyrosinemia and the Fanconi syndrome. Succinylacetone inhibits rat renal tubular concentrative uptake of the glucose transport analogue, methyl α-D-glucoside, in a noncompetitive and reversible fashion. This compound also depresses oxygen consumption by the rat renal tubule without fine structural damage to mitochondria. It is concluded that succinylacetone may be a useful probe in elucidation of the biochemical mechanism underlying the human Fanconi syndrome.

Introduction

Hereditary tyrosinemia is an autosomal recessive disease resulting in hepatorenal dysfunction. The renal manifestations of the disease include renal tubular acidosis of the proximal variety, aminoaciduria, glycosuria and phosphaturia, comprising the entity known as the Fanconi syndrome [1]. The biochemical basis for the disease was determined in 1977, following the description by Lindblad et al. [2] of the urinary excretion of succinylacetoacetate and succinylacetone by affected individuals. Numerous studies by various investigators have confirmed a deficiency of fumarylacetoacetate hydrolase activity in liver from tyrosinemic patients [3–5]. Thus, the elevated blood tyrosine and the urinary tyrosine metabolites seen in this disease can be adequately explained on the basis of the enzymatic defect. However, the mechanism underlying the accompanying Fanconi syndrome has remained speculative. On the basis of a single study in vivo [6], most investigators have assumed that the succinylacetoacetate and succinylacetone produced as a result of the enzymatic defect are inhibitors of renal tubular function. This hypothesis has not yet been examined directly in vitro, to the best of our knowledge.

The possibility that a catabolic intermediate of tyrosine might induce renal tubular dysfunction, as well as our long-standing interest in the maleic acid-induced animal model of the human Fanconi syndrome [7,8], led us to an investigation of the effects of succinylacetone on rat renal tubular transport of methyl α-D-glucoside, a glucose transport analogue [7]. The results of this study, reported in the present paper, suggest the basis for a new, more physiological animal model of the human renal tubular transport dysfunction known as the Fanconi syndrome.

Methods and Materials

Male rats of the Sprague-Dawley strain, weighing 150–175 g, were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals

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were provided water and Purina chow ad libitum. Methyl α-D-[U-^{14}C]glucoside (275 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Unlabeled methyl α-D-glucoside was purchased from Sigma Chemical Co. (St. Louis, MO). Succinylacetone was obtained from Calbiochem (San Diego, CA), made to 200 mM stock in water (pH 7.0) and stored at −20°C until used. Collagenase, grade II, was obtained from Worthington Biochemical Corporation with a specific activity of 155–170 units/mg. Fetal calf serum was obtained from Flow Laboratories, divided into 10-ml aliquots and stored frozen until used, to prevent bacterial growth.

In vivo studies

Animals were weighed and housed individually in metabolic cages; each rat was given free access to food and water. Following an overnight period of acclimatization, urine was collected for a 24-h period and frozen at −20°C. The animals were then injected intraperitoneally with neutralized (pH 7.0) succinylacetone; the dose was based upon the assumption that succinylacetone is equally distributed in total body water and calculated to achieve a 4 mM concentration in blood. Controls were given equal volumes of normal saline intraperitoneally. Urines were collected over the subsequent 24-h period and frozen at −20°C until analyzed. Amino acid quantitation was performed using a JEOL JLC-5AH automated amino acid analyzer.

Renal cortical and hepatic ATP levels were measured enzymatically using a kit purchased from Sigma Chemical Company (St. Louis, MO). Animals were injected with normal saline or sufficient succinylacetone to achieve 4 mM blood concentration, and were killed by stunning and decapitation 2 h later. Liver and kidneys were rapidly removed and placed in iced Krebs-Ringer bicarbonate buffer. Cortical slices were made using a Stadie-Riggs microtome and the tissues were homogenized. Protein concentrations were measured by the Lowry method and the results expressed as μmol ATP/mg protein.

Determination of sugar accumulation

Isolated renal tubules were prepared by collagenase digestion of minced cortical slices, as previously described [7]. The tubules were washed three times, filtered through two layers of gauze and resuspended in Krebs-Ringer bicarbonate buffer, pH 7.4. Final volumes of suspension were between 20 and 60 ml, with addition of 1 ml of fetal calf serum per 20 ml of suspension. Each milliliter of incubation mixture contained 0.1 μCi of methyl α-D-glucoside, with unlabelled sugar added to achieve the desired final concentrations. The experimental method for determining uptake of methyl α-D-glucoside by tubules has been described by us and others in detail [7–9]. Briefly, the tubules are gassed with 95% O_2/5% CO_2 in a special flask [9] at 37°C and serial samples are removed at various times; these are centrifuged at 33000 × *g*. The supernatant and pellet are separated and assayed for radioactivity. 'Trapped medium' space and total water were 15.2% and 80%, respectively [7]. Tissue substrate concentrations and distribution ratios (cpm/ml intracellular fluid to cpm/ml medium) were determined as previously described [10].

Steady-state kinetic studies

In order to determine the amount of substrate entering the tubular cell per unit time, the suspension was incubated with and without 4 mM succinylacetone in the presence of 2 mM unlabelled sugar for sufficient time to achieve a steady-state distribution ratio. After 30 min of preincubation, 0.1 μCi of tracer was added per ml of suspension and samples were taken and treated in a fashion identical to that used for uptake studies. Fractional rate constants were calculated by the method of Rosenberg et al. [11] for a two-compartmental system, as employed by McNamara et al. [12].

Concentration-dependence studies

The tissue was preincubated with or without 4 mM succinylacetone for 30 min in Krebs-Ringer bicarbonate buffer, at which time 0.1 μCi of tracer per ml of suspension plus sufficient unlabelled sugar to give the desired final concentration were added. Samples were taken at 5 min and the distribution was ratios calculated. A double-reciprocal plot was made to assess the effect of 4 mM succinylacetone on the V_{max} and apparent transport K_m. Lines were drawn using a least-

squares method of analysis. All data were analyzed for significance by Student's *t*-test.

Oxygen-consumption studies

Tubules were prepared as described above and 1 ml was suspended in 2 ml reaction buffer containing 0.32 M mannitol, 4 mM potassium phosphate, 4 mM KCl, 0.37 mM EDTA. The suspensions were either untreated or treated with 4 mM succinylacetone. Oxygen consumption was measured polarographically at 30°C with a Clark fixed-voltage electrode. Substrates, when added (fortified respiration), were included as 7 mM succinate, 7 mM glutamate, plus 5 mM glucose. Results are expressed as nmol oxygen/min per ml suspension.

Results

In vivo effects of succinylacetone on renal tubular function and ATP levels

Although 24-h urinary volume did not differ significantly during pre- and post-injection periods, administration of succinylacetone intraperitoneally resulted in proteinuria (> 300 mg/dl) and glucosuria (> 250 mg/dl). Quantitative measurement of urinary amino acids revealed increases in several following succinylacetone injection (Table I). Tissue ATP levels 2 h after succinylacetone injection did not vary significantly from controls.

TABLE I

URINARY AMINO ACID EXCRETION BY SUCCINYLACETONE-TREATED RATS

Animals were handled as described in Methods and Materials. Amino acids were separated and quantitated on an automated amino acid analyzer. Only those mean values (±standard errors) which were consistently and significantly elevated are included.

Amino acid	Urinary excretion (μmol/24 h)	
	control	succinylacetone-treated
Ornithine	0.20±0.075	0.50±0.045
Aspartate	0.79±0.163	3.56±0.326
Threonine	0.95±0.347	2.67±0.298
Glutamine	4.25±0.857	7.99±0.975
Valine	0.30±0.080	0.70±0.046
Methionine	0.11±0.071	0.50±0.022

Effects of succinylacetone on concentrative uptake

The steady-state distribution ratio achieved by the tubules for methyl α-D-glucoside was significantly depressed ($P < 0.001$) in the presence of 4 mM succinylacetone, compared to controls (Fig. 1A). Any further increase in concentration of the inhibitor to 8 mM did not produce a proportionately greater effect on the steady-state concentration gradient achieved. The inhibitory effect of succinylacetone was exerted fully by 30 min of incubation, by which time both control and experimental tissue achieved and maintained steady-states. Preincubation with 4 mM succinylacetone for 30 min did not further increase the difference in steady-state levels achieved (Fig. 1B). Initial rates of uptake in the presence of succinylacetone appeared to differ from controls.

The effect of 4 mM succinylacetone on the integrity of the tissue was evaluated in several independent ways. Tubules were incubated with and without 4 mM succinylacetone under conditions identical to those used for uptake studies. These tubules were then fixed and examined by electron microscopy. No differences were observed between control tissue and tubules exposed to 4 mM succinylacetone for thirty minutes (Fig. 2). In another series of experiments, tubules were incubated for 30 min in three separate flasks, two of

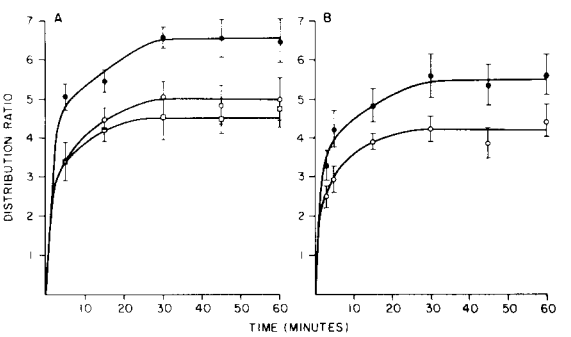

Fig. 1. Effect of succinylacetone on uptake of methyl α-D-glucoside. (A) Tubules were incubated alone (●) or with 4 mM (○) or 8 mM (□) succinylacetone as described in text. (B) Tubules were preincubated for 30 min alone (●) or with 4 mM succinylacetone (○) prior to addition of substrate. All points represent the means + standard errors of 9–12 separate determinations. Uptake is plotted as the distribution ratio (cpm per ml intracellular fluid : cpm per ml medium) achieved versus time (in minutes).

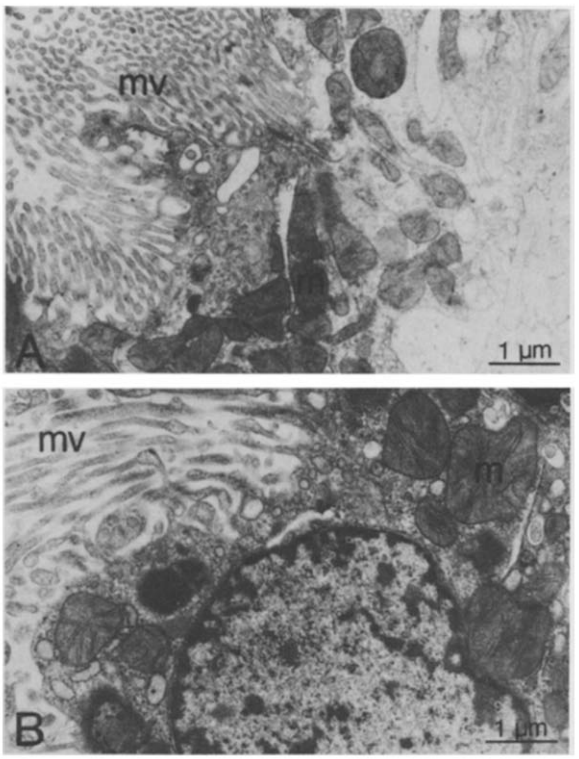

Fig. 2. Electron micrographs of tubules incubated for 30 min at 37°C with continuous gassing with 95% O_2/5% CO_2 without (A) and with (B) 4 mM succinylacetone. Upon completion of control or experimental incubation, samples were centrifuged at 3000 rpm for 10 min to form a loose pellet. Pellet were fixed for 2 h at 4°C in 1.0% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Following aldehyde fixation, samples were postfixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2), stained en bloc in 0.5% uranyl acetate in 0.5 M maleate buffer (pH 5.2) for 24 h, dehydrated in methanols and propylene oxide, and embedded in TAAB 812 plastic resin. Ultrathin sections were cut with a diamond knife on an LKB Ultratome IV ultramicrotome, collected on Formvar-coated single-slot grids, stained with uranyl acetate (4% in absolute methanol, 15 min) and lead citrate (0.1% in 0.1 M NaOH, 2 min), and examined and photographed with a Zeiss EM-10CA electron microscope. Note comparatively similar appearance of microvilli (mv) and internal (cristae) and external membranes of mitochondria (m). Differences in size of mitochondria reflect different regions of the proximal tubule from which the photographs were obtained. Instrument magnification: ×22500.

which contained 4 mM succinylacetone. At the end of this incubation, the tissue was separated from the original buffer and resuspended in buffer alone (flasks 1 and 2) or buffer +4 mM succinylacetone (flask 3). Substrate was then added and an uptake experiment was carried out as described above. No significant difference ($P > 0.05$) in uptake of methyl α-D-glucoside was observed between control tubules and those exposed to 4 mM succinylacetone and resuspended in buffer alone. On the other hand, continued exposure to succinylacetone resulted in inhibition of methyl α-D-glucoside uptake, thus demonstrating the reversibility of the transport effects of succinylacetone (Fig. 3). Finally, oxygen consumption by the tubules was examined with and without 4 mM succinylacetone after a 30-min preincubation. Succinylacetone treatment reduced oxygen consumption compared to controls by 50% in the presence of endogenous substrate alone (controls = 82.8 nmol O_2/min per ml versus 40.0 nmol O_2/min per ml) on five separate determinations. A similar relationship between control and succinylacetone-treated tubules pertained with fortified respiration (115.4 versus 76.1 nmol O_2/min per ml) on four separate determinations).

Steady-state kinetic effect of succinylacetone

Addition of tracer following a 30-min prein-

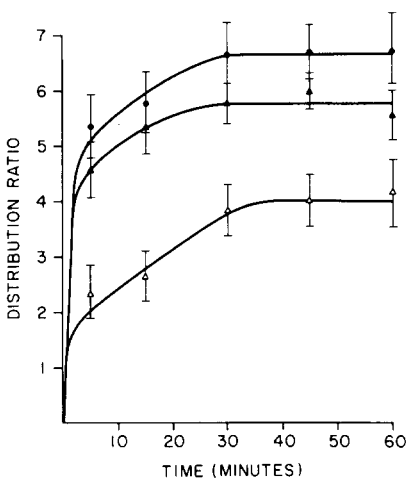

Fig. 3. Reversibility of the effect of 4 mM succinylacetone on renal tubular uptake of 2 mM methyl α-D-glucoside. Tubules were incubated alone (●) or with 4 mM succinylacetone (▲, △) for 30 min as described in the text. The tissue in each flask was separated from the original buffer and resuspended in buffer alone (●, ▲) or in buffer+4 mM succinylacetone (△). 2 mM methyl α-D-glucoside was added to each flask and an uptake experiment was performed. Each point represents the mean ± standard error of 9–12 separate determinations.

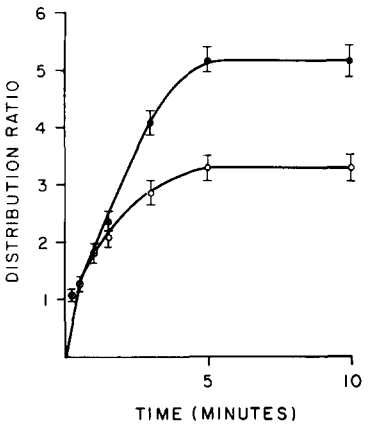

Fig. 4. Steady-state kinetic effects of succinylacetone. Tubules were incubated as described in the text for 30 min with 2 mM unlabelled methyl α-D-glucoside in buffer alone (●) or buffer + 4 mM succinylacetone (○). Tracer was added after preincubation and an uptake study was performed. Points represent the mean ± standard error of 9–12 separate determinations.

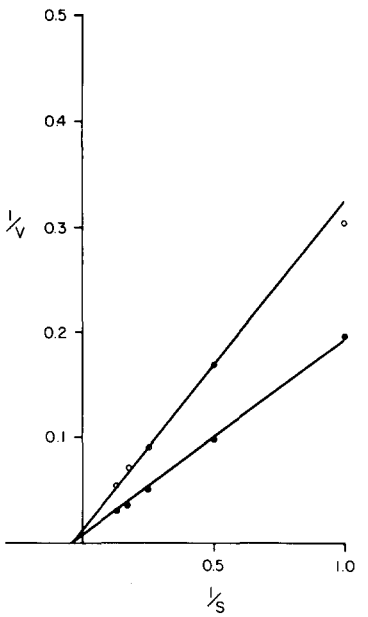

Fig. 5. Effect of succinylacetone on concentration-dependent uptake. Tubules were preincubated for 30 min in buffer (●) or buffer + 4 mM succinylacetone (○). After preincubation tracer (0.1 μCi/ml) plus unlabelled sugar were added to give the desired concentrations. Incubation was carried out for 5 min, and samples were removed and treated as described previously. Points represent means of 9–12 separate determinations and lines were fitted by the least-squares method. S is the substrate concentration expressed in mmol/l and V is the velocity of uptake in nmol/liter intracellular fluid per 5 min.

cubation with unlabelled substrate to allow establishment of a steady-state demonstrated a pronounced slowing of initial uptake of methyl α-D-glucoside by 4 mM succinylacetone (Fig. 4). Steady-state kinetic parameters calculated from these experiments are shown in Table II.

Effect of succinylacetone on concentration-dependent uptake

Uptake of methyl α-D-glucoside was noncompetitively inhibited by 4 mM SA (Fig. 5). The apparent transport K_m for both control and experimental tissue was 24 mM, while the transport V_{max} values were 160.3 and 80 nmol/l per 5 min, respectively.

TABLE II

STEADY-STATE KINETIC PARAMETERS OF METHYL α-D-GLUCOSIDE TRANSPORT

Triplicate experiments were carried out at 37°C as described in the text. All calculations are based on 100 mg of tissue. Statistical analysis was performed using Student's t-test. Fractional rate constants are expressed as the mean values ± standard error.

$$\text{medium} \underset{\lambda_{MI}}{\overset{\lambda_{IM}}{\rightleftharpoons}} \text{intracellular pool}$$

Condition	Steady-state distribution ratio	Intracellular pool (μmol)	Fractional turnover rate (min^{-1})	
			λ_{IM}	λ_{MI}
Control	5.20	0.7018	0.044 ± 0.006	0.636 ± 0.0121
Succinylacetone (4 mM)	3.30	0.4454	0.027 ± 0.003	0.608 ± 0.0130

Discussion

The biochemical basis for the human Fanconi syndrome has not been elucidated. In order to further the understanding of this phenomenon, we have utilized succinylacetone to produce an animal model for study. The rationale for use of succinylacetone derives from the postulate that the renal tubular dysfunction in hereditary tyrosinemia is causally related to the presence of succinylacetone and its immediate precursor succinylacetoacetate [6]. Our observations lend support to this speculation and constitute the first such evidence in an animal model.

The in vivo effects of succinylacetone on renal tubular function, including proteinuria, glucosuria and amino aciduria, are suggestive of a renal Fanconi syndrome. The identities of the specific urinary amino acids indicate an effect of succinylacetone on basic, neutral and acidic transport systems, thus representing a generalized disruption of renal tubular amino acid transport. The appearance of glucosuria with succinylacetone administration is important to the present work since it documents the relevance of in vitro characterization of sugar uptake inhibition by succinylacetone. The absence of a demonstrable succinylacetone effect on renal cortical and hepatic ATP levels is most likely attributable to a reversible depression in oxygen consumption caused by succinylacetone. The effects of succinylacetone on isolated kidney mitochondria are currently under investigation.

We were able to demonstrate marked inhibition of methyl α-D-glucoside uptake by the renal tubule in the presence of 4 mM succinylacetone. This effect was immediate in onset and was reflected by a substantial decrease in oxygen consumption. On the other hand, no fine structural damage to mitochondria was caused by succinylacetone, consistent with the reversibility of transport inhibition which we observed. The proportionately diminishing inhibition in methyl α-D-glucoside transport with increased succinylacetone concentration above 4 mM is suggestive of succinylacetone saturability of the affected energy-generating system for active transport.

The likelihood that the effect of succinylacetone on membrane transport is indirectly mediated through an effect on energy generation is further supported by our observation that it causes a depression of the initial rate of uptake in a noncompetitive fashion. It has been previously demonstrated that compounds which act upon oxidative phosphorylation cause a noncompetitive type of transport inhibition and a diminished substrate influx [7,11,14,15].

Many of the present observations regarding the actions of succinylacetone on rat renal tubule are directly analogous to those of maleate. The latter compound has been shown to reversibly inhibit concentrative uptake of methyl α-D-glucoside without causing fine structural alterations in rat renal tubular cells in vitro [7]. Rogulski et al. [15] have examined the effects of maleate on oxidative metabolism in rat kidney mitochondria, observing a decrease in both oxygen uptake and phosphorylation and concluding that substrate level phosphorylation is inhibited. While decreased oxygen consumption by the intact tubules in the presence of 4 mM succinylacetone does not prove a similar mechanism, it is consistent with such an explanation. Studies utilizing isolated renal tubular mitochondria with succinylacetone are currently in progress in an effort to establish the mechanism(s) involved.

Since succinylacetone is a metabolic end-product found in large quantities in humans who suffer from hereditary tyrosinemia and the Fanconi syndrome, in vitro examination of the effects of this compounds on renal tubular transport and metabolism may be important to an understanding of the biochemical events underlying the renal tubular dysfunction of human kidney. The results presented here support the hypothesis that succinylacetone is an endogenously produced renal tubular transport inhibitor which may be responsible for the association of the Fanconi syndrome with hereditary tyrosinemia in humans.

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