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Renal heme metabolism in hereditary tyrosinemia: use of succinylacetone in rat renal tubules

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Succinylacetone (SA), a metabolic end-product found in urine from individuals with hereditary tyrosinemia and associated renal Fanconi syndrome and a known inhibitor of hepatic 5-aminolevulinic acid dehydratase (ALAD), has been used to study heme metabolism in isolated rat renal tubules. Heme biosynthetic porphyrin precursors are increased selectively in the presence of 4 mmol/l SA. Total porphyrin content of the tubules are increased approximately 2-fold, while both ferrochelatase and heme oxygenase activities remain unaffected by SA. Nonetheless, total heme content is reduced, as was incorporation of radioactive label from amino[^{14}C]levulinic acid. Cytochrome *P*-450 content remained unaffected. Impairment of iron uptake and/or transport within the cell or enhancement of heme catabolism via a non-heme oxygenase-dependent pathway could explain the observations.

Introduction

Hereditary tyrosinemia, an autosomal recessive disorder due to defective enzymatic cleavage of fumarylacetoacetate, results in accumulation and urinary excretion of succinylacetone [1,2]. Succinylacetone (SA) has been successfully used to generate an animal model for the renal Fanconi syndrome [3], a generalized tubular dysfunction, which is associated with the genetic disorder in humans. Hereditary tyrosinemia is also known to cause increased urinary excretion of 5-aminolevulinic acid, an intermediate of heme biosynthesis. Moreover, SA is a potent inhibitor of 5-aminolevulinic acid dehydratase (ALAD) in cultured chick hepatocytes [4], human red cells [5], and rat liver [6]. Thus there appears to be a relationship between the inherited biochemical defect and the inhibitory action of the resulting biochemical marker compound on heme metabolism. In a recent study, the effects of SA on renal tubular ALAD were examined and found to be very different from those seen with crude liver homogenate [7]; ALAD activity was enhanced in a concentration-related fashion, leading to the expectation that subsequent products, notably heme, might also be enhanced in the presence of SA. These relationships are shown in Fig. 1. In view of the increased hepatic production of 5-aminolevulinate known to occur in the presence of SA [8], enhancement of renal aminolevulinate uptake coupled to increased ALAD activity leading to compensatory heme synthesis by the kidney could explain the observations.

Uptake of 5-aminolevulinate in the presence of SA, however, is significantly impaired in both isolated renal tubules and brush-border membrane vesicles [9]. Moreover, despite the increase in ALAD activity observed in the renal tubule, incorporation of radioactive label into heme from 5-amino[^{14}C]levulinate was diminished. In order to examine this latter observation more closely, it was necessary to study the formation and breakdown of heme. Thus, we have quantitatively measured the effects of SA on heme and porphyrin formation by isolated renal tubules, ferrochelatase activity in renal mitochondria and heme oxygenase activity in microsomes prepared from isolated renal tubules, as well as

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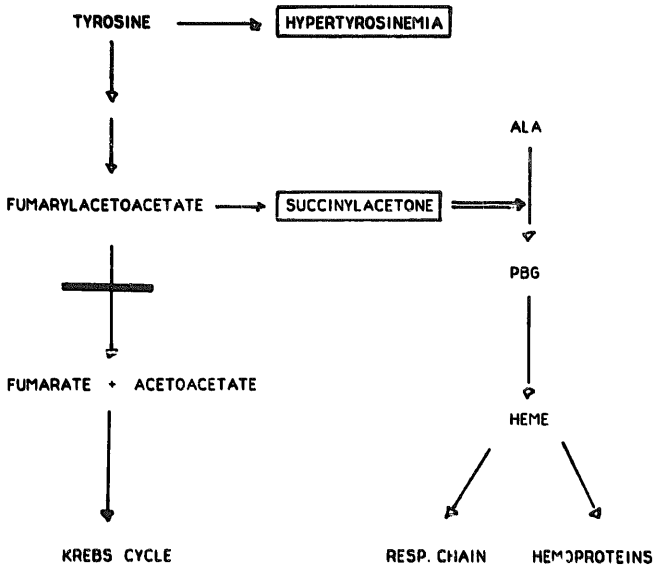

Fig. 1. Relationship of the metabolic defect in hereditary tyrosinemia to heme biosynthesis. Expression of the mutant gene in liver and kidney results in impaired activity of fumarylacetoacetate hydrolase, leading to production of succinylacetoacetate and succinylacetone. The latter compound is a potent inhibitor of aminolevulinc acid dehydratase in liver, and is known to impair hepatic heme synthesis as a consequence of this inhibitory property. However, succinylacetone enhances the dehydratase activity in renal tubules, simultaneously reducing the apparent rate of label incorporation from ALA into heme.

total cytochrome *P*-450 in these tubules. The results of these studies suggest an entirely new basis for our previous observations.

Methods and Materials

Adult male Sprague-Dawley rats (150–200 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Animals were provided food and water ad libitum, until they were killed by stunning and decapitation. Kidneys were rapidly removed and placed into ice-cold saline. Cortical slices were made with a Stadie-Riggs microtome and isolated tubules prepared as previously described [3]. In all experiments, tissue was prepared in a single batch and divided into control and experimental fractions in order to avoid preparative differences influencing our results. All subsequent steps were carried out in subdued lighting and with shielded vessels to prevent photoconversion of porphyrins. All incubation and preparative buffers used for experimental samples contained 4 mmol/l SA. After a 60-min incubation in Krebs-Ringer bicarbonate buffer with continuous gassing [3], control and experimental tubules were separated from the buffer by low speed centrifugation. The pellets were surface-dried and weighed. The tissue was then diluted with 4 vol. of buffer (100 mmol/l Tris, 100 mmol/l KCl, 1 mmol/l EDTA, 20 μmol/l butylhydroxytoluene, pH 7.4) and sonicated for 35 s. The solutions were then centrifuged for 60 min at 105000 $\times g$ in a Beckman model L50 preparative ultracentrifuge. The resulting supernatants were cytosols which were removed quantitatively for porphyrin determinations. Microsomal heme was removed from the pellet by resuspension in 4 vol. of a wash buffer (0.1 mol/l sodium pyrophosphate, 1 mmol/l EDTA, pH 7.5) and recentrifugation at 105000 $\times g$ for 60 min. Supernatants were then discarded and the pellets resuspended in 2 ml of 'microsome buffer' (100 mmol/l potassium phosphate, 1 mmol/l EDTA, 20% glycerol, 1 mmol/l DTT, 20 μmol/l butylhydroxytoluene, pH 7.25) and homogenized. The homogenization vessel was rinsed with an additional 2 ml of buffer and combined. While samples may be stored in this solution at −70 °C, we assayed heme oxygenase and cytochrome *P*-450 in the present study on the day of preparation. Proteins were determined by the Bio-Rad method (Richmond, CA). Microsomal heme oxygenase and cytochrome *P*-450 were measured spectrophotometrically [10,11]; the data were expressed as nmol/min per mg protein or nmol/mg protein, respectively. Other samples of tubules were stored at −70 °C from which total heme was extracted [9] and assayed spectrophotometrically [12].

Cytosolic porphyrins were determined by reversed-phase high performance liquid chromatography, using a 4.5 mm × 25 cm C18 Ultrasphere ODS column and a 45 mm guard column, on a HP1090 chromatograph equipped with a DR 5 solvent delivery system and a Model 7012 Rheodyne injector with a 250 μl loop. Detection was performed using a Gilson 121 fluorescence detector, equipped for excitation at 398 nm and emission at 620 nm, with a 9 μl flow cell. Aqueous solvents were prepared using deionized water. A 0.1 mol/l (11.503 g/l) monobasic ammonium phosphate solution was adjusted to pH 3.5 using concentrated phosphoric acid and filtered through a 0.45 μm pore Millipore filter [13]. Methanol was added to provide a final ratio of 56 parts ammonium phosphate solution to 44 parts methanol [14]. The pH of the mixture (Solvent A) was adjusted to 3.4 with phosphoric acid, stored at room temperature and degassed with helium for 10 min prior to use. Solvent B was pure methanol. 1-ml aliquots of cytosols were adjusted to pH 2.5 with 5 mol/l HCl, vortexed for 10 s and centrifuged at 15000 rpm for 5 min. From the supernates 225 μl were removed and combined with 25 μl (4 ng) of mesoporphyrin standard for injection. Mesoporphyrin and protoporphyrin stock solutions (16 mg/l) were prepared by dissolving 1.6 mg in 0.5 ml 5 mol/l HCl, followed by addition of 90 ml deionized water. The solutions were adjusted to pH 2.0 with 0.1 mol/l HCl and further diluted to 100 ml final volume with deionized water. Other standards, prepared from a kit, were made up by addition of one drop of 5 mol/l HCl to the vial, followed by 30 min of standing at room temperature.

The vial was then vortexed, 7 ml of 0.1 mol/l HCl added, the pH adjusted to 2.0, and the volume brought to 8 ml with deionized water to yield final concentrations of each component of 1.25 nmol/ml. Chromatographic conditions were as follows: an 18-min gradient was employed to increase solvent B from 50 to 100% (Lambrecht, R., personal communication). This gradient was then reversed to re-equilibrate the column. A second (rinse) gradient was run without injection to clean the column of porphyrins. Both pumps had a combined flow rate of 1.5 ml/min. All porphyrins were eluted within the 18 min run time, and were identified and quantitated against the known standards. Data were expressed as ng individual and total porphyrin per mg protein.

Mitochondria were prepared from whole rat kidney, using a previously published method [15], and the final pellets resuspended in 0.05 mol/l Tris (pH 7.5), 1 : 1 (v/v). Ferrochelatase activity was measured spectrophotometrically, with and without added 4 mmol/l succinylacetone, as previously described [16,17]. Data were expressed as nmol mesoheme produced/h per mg mitochondrial protein.

Authentic porphyrins and porphyrin acid chromatograpic marker kits were obtained from Porphyrin Products (Logan, UT). Succinylacetone was obtained from Calbiochem (San Diego, CA). All other reagents were purchased from commercial sources and were of the highest degree of purity available.

Results

Measurement of total cytosolic porphyrins showed a marked increase with addition of 4 mmol/l succinylacetone during the 60 min incubation. A representative chromatogram is shown in Fig. 2. Specifically, while proto- + uroporphyrin in controls accounted for 56.3% of total porphyrins, these two compounds represented 70.2% of the total in the treated tissue. Of the total increment of change in porphyrins with SA, the changes in these two tetrapyrroles together accounted for 89.1%. While intermediates of conversion from uroporphyrin to coproporphyrin (penta-, hexa- and heptaporphyrins) were increased, as well, the differences were not significant. These data are summarized in Table I.

Elevated levels of all porphyrins in the heme biosynthetic pathway led us to examine inhibition of the final enzyme step. Since SA is not known to be further metabolized in mammalian cells, we took advantage of this fact in measuring ferrochelatase activity in isolated renal mitochondria. This activity in SA-treated mitochondria did not differ from controls, despite the increase in porphyrin substrate. Nonetheless, total heme content of SA-treated cells was reduced significantly below controls. Activity of the primary heme catabolic

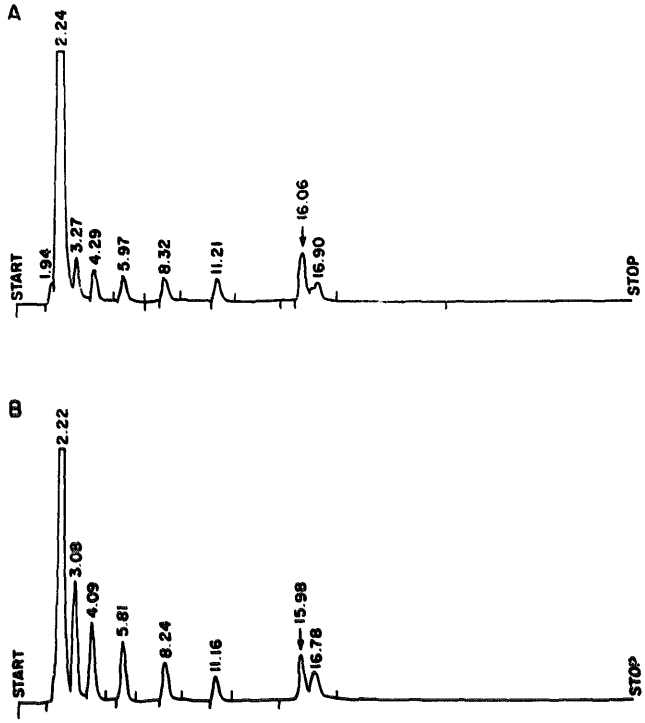

Fig. 2. Representative chromatograms of renal cytosolic porphyrins. Renal tubules were incubated as controls (A) and with 4 mmol/l succinylacetone (B) as described in Methods. Conditions for separation of cytosols and porphyrin separation and detection are also detailed in Methods. After the initial solvent front, peaks appear in the following order: uroporphyrin, heptaporphyrin, hexaporphyrin, pentaporphyrin, coproporphyrin, mesoporphyrin (internal standard), and protoporphyrin. The protein content of the samples was equivalent; control 13.2 mg/ml, experimental 13.9 mg/ml.

enzyme, microsomal heme oxygenase, did not change from control in the presence of SA. The values, though quite low, are consistent with those reported previously by other investigators [18]. These data are summarized in Table II. Cytochrome *P*-450, accounting for a size-

TABLE I

Effects of succinylacetone on renal tubular cytosolic porphyrin profile

Individual porphyrins were measured by fluorescence and identified against authentic standards, following separation by HPLC as detailed in Methods. Values shown represent the means (±S.E.) of three separate experiments, each sample analyzed in triplicate. Data are expressed as ng porphyrin/mg tubule protein. Significant differences (*) induced by SA exposure, were determined by Student's '*t*-test' ($P < 0.05$). Hepta-, penta- and hexaporphyrins are trivial names for differently-substituted porphyrin ring structures measured by our technique.

Porphyrin	Control	Control + 4 mM SA
Proto-	6.23 (±0.93)	10.57 (±1.12) *
Hepta-	4.16 (±0.38)	4.57 (±0.62)
Uro-	1.83 (±0.26)	6.90 (±1.03) *
Penta-	0.63 (±0.21)	0.89 (±0.30)
Copro-	0.72 (±0.34)	0.60 (±0.19)
Hexa	0.75 (±0.27)	1.34 (±0.31)

TABLE II

Effects of succinylacetone on the heme biosynthetic pathway

Determinations of each parameter shown was performed as described in Methods. Values shown represent the means (± S.E.) of at least three separate experiments. Data for radioactive heme ([a]) from a previous report [9] are included here, as determined for incorporation of label from ^{14}C-aminolevulinate, for comparison. Results were examined for significance by Student's '*t*-test'; asterisks(*) denote $P < 0.05$.

	Total porphyrins (ng/mg protein)	Ferro-chelatase (nmol/h per mg)	Radioactive heme [a] (nmol/h per mg)	Heme oxygenase (nmol/min per mg)	Total heme (nmol/mg)
Control	14.32 (±1.59)	2.48 (±0.116)	0.457 (±0.043)	0.0034 (±0.0002)	1.86 (±0.60)
Control +4 mM SA	24.87 * (±1.89)	2.36 (±0.163)	0.101 * (±0.011)	0.0029 (±0.0004)	0.64 (±0.31)

able portion of total cellular heme also remained unaffected by SA (data not shown).

Discussion

The biosynthesis of heme can be viewed as a process requiring two substrates (iron + porphyrin) and resulting in the single product, heme. In the present report we have examined the process by which porphyrin is produced in the renal tubular cell, since it is this pathway which has been identified as abnormal in various mammalian tissues with SA exposure [1–3]. The basis for the abnormality has been demonstrated to be due to competitive inhibition of ALAD in liver [4]; however, we have previously reported a significant increase in renal tubular ALAD activity induced by SA [7]. Despite this increase, however, we found a decreased incorporation of radiolabeled ^{14}C-ALA into heme in a subsequent study [9]. Thus, the enhanced ALAD activity caused by SA appeared to be offset by some other effect, an observation which led to the present studies.

Woods [19] has reported activites of six enzymes of the heme biosynthetic pathway in male Sprague-Dawley rat kidney cortex. Of these, ALAD and ferrochelatase show the highest specific activites, in each case the level being approximately one order of magnitude greater than the other four enzymes. Our present demonstration that heme porphyrin precursors are very significantly increased within the cytosolic fraction of the tubule cell is entirely consistent with the SA-enhanced activity of ALAD. The fact that increased protoporphyrin, the immediate precursor of heme, could be found within these cells strongly suggests the absence of any SA effect on the intervening enzymatic steps between PBG and protoporphyrin formation. It is possible, in view of the marked increase in uroporphyrin observed, that SA exerts an inhibitory effect on uroporphyrinogen decarboxylase (UROD), as has been demonstrated for other porphyrinogenic compounds [20–22]. Such an effect, however, would be partial at best, since coproporphyrin levels were not significantly reduced, and activity of UROD in human liver is roughly equivalent to that of coproporphyrinogen oxidase, the next enzyme of the sequence [19,23,24].

Total heme reduction with SA treatment in the present studies is entirely consistent with the earlier report of diminished ^{14}C-incorporation into heme [9]. These findings could be explained as a consequence either of decreased synthesis through the ferrochelatase step, or from enhanced breakdown. It is clear from our data that the capacity for renal mitochondrial ferrochelatase to incorporate iron and porphyrin into heme remains unaffected by 4 mmol/l SA, when measured under optimal conditions. This would suggest that in the presence of large quantities of protoporphyrin heme synthetic rate in SA-treated tubules should be at least equivalent to that of control tubules. The absence of any demonstrable effect of SA on heme oxygenase in the renal tubule, as well as the equivalence of microsomal cytochrome *P*-450 in both control and treated tissue lend support to the view that experimentally-reduced heme does not result from enhanced heme catabolism.

Either availability of iron as the factor limiting apparent ferrochelatase activity, or enhancement of heme catabolism by SA through a pathway not requiring heme oxygenase may be invoked as an explanation for our findings. Our earlier observations regarding decreased label incorporation into heme were performed under conditions including a large surplus of iron-transferrin [9]. This does not, however, exclude the possibility that iron-availability is the limiting factor in our system. Mechanisms responsible for movement of iron from the cell surface to mitochondrial surface membranes have not yet been delineated, although this transport is clearly essential to normal ferrochelatase function.

The alternative possibility, that enhancement of heme catabolism, coupled with increased heme synthesis might explain our findings, is more problematic. Since the existence of a non-heme oxygenase dependent pathway has been documented [25,26] but remains completely uncharacterized, it is not possible to

fully evaluate the contribution of this pathway to our results. However, we have clearly shown that catabolism of heme via heme oxygenase remains unaffected in the presence of 4 mmol/l SA.

In summary, then, the present studies show that aside from the previously reported enhancement of ALAD activity in the renal tubule, SA apparently has no major effect on the subsequent steps of the heme biosynthetic pathway. Moreover, measurable heme catabolic activity is also unaffected. Thus, we suggest that the overall reduction in total heme content derives from a diminished iron supply in the face of normal ferrochelatase activity. An effect of SA on the poorly-characterized, heme oxygenase-independent heme catabolic pathway could also help to explain an apparent decrease in label incorporation into heme, and together with increased ALAD activity, this mechanism could account for our present observations. In view of the above, SA could prove to be a valuable probe in further delineation of renal tubular iron metabolism.

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