Effect of succinylacetone on heme and cytochrome P450 synthesis in hepatocyte culture

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The effects of succinylacetone, a tyrosine metabolite, on the hepatic biosynthesis of heme and cytochrome P450 were studied in primary culture of chick embryo hepatocytes. Succinylacetone potentiated the phenobarbital-mediated induction of δ-aminolevulinate synthase, strongly inhibited porphobilinogen synthase activity, reduced cellular heme concentration and impaired induction of cytochrome P450. Enhanced induction of δ-aminolevulinate synthase and decreased cytochrome P450 induction may be explained by the succinylacetone-mediated inhibition of porphobilinogen synthase and the subsequent depletion of intracellular heme, since these effects of succinylacetone were reversed by addition of heme. These results suggest clinical implications for patients with tyrosinemia, who accumulate succinylacetone.

Succinylacetone *Heme* *Cytochrome P450* *δ-Aminolevulinate synthase* *Porphobilinogen synthase* *Tyrosinemia* *Hepatocyte culture*

1. INTRODUCTION

Succinylacetone (SA, 4,6-dioxoheptanoic acid), a metabolite of tyrosine, accumulates in patients with hereditary tyrosinemia presumably due to a decreased activity of fumarylacetoacetase [1]. Recent studies suggest that SA is a potent, irreversible inhibitor of porphobilinogen (PBG) synthase (EC 4.2.1.24), the second enzyme of heme synthesis, by binding to the active site of the enzyme [2]. The inhibition of PBG synthase by SA in patients with tyrosinemia leads both to excessive accumulation and excretion of δ-aminolevulinate (ALA) and is associated with the occurrence of neurological symptoms characteristic of acute attacks of hepatic porphyria. Thus, the defect in hereditary tyrosinemia seems to alter yet another important metabolic pathway, the biosynthesis of heme [1].

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This study was designed to examine the effects of SA on the biosynthesis of heme and hemoproteins in the liver. Heme synthesis is mainly controlled by negative-feedback regulation of the first enzyme of the pathway, ALA synthase (EC 2.3.1.37) [3]. In the liver > 60% of the heme synthesized is required to maintain cytochrome P450 hemoprotein concentration, the key component of the microsomal drug oxidation system. Moreover, induction of cytochrome P450 hemoproteins by drugs and other chemicals is associated with an increase in hepatic ALA synthase activity and heme biosynthesis presumably by decreasing a regulatory cellular heme pool [3–5]. We have used chick embryo hepatocytes cultured under chemically defined conditions. This culture system uniquely preserves inducibility of ALA synthase [3] and cytochrome P450 hemoproteins by drugs, such as phenobarbital [6–8].

2. EXPERIMENTAL

Hepatocytes were isolated from 15-day-old

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chick embryos (Shaver hybrids) by in situ perfusion of the liver and collagenase digestion, and 1.5×10^7 cells were cultured in 10 ml serum-free Williams' E medium containing 2.5 mM glucose and 1.5 μM triiodothyronine in 10 cm Falcon culture dishes for 44 h as in [8]. The medium was changed after 24 h culture. SA dissolved in medium (neutralized and filtered through 0.22 μm millipore filters) was present for the last 20 h culture and 0.4 mM phenobarbital was added for the final 16 h culture (induction period). Heme was dissolved in 0.25% sodium carbonate (pH 8.0) and was administered twice, 2 h before and 4 h after the addition of phenobarbital to reach 3 μM final conc.

For the preparation of homogenate and microsomes, hepatocyte monolayers from 3 culture dishes were washed twice with 0.25 M sucrose, scraped off the dishes, homogenized and sonicated in 2 ml sucrose at 4°C as in [8]. Microsomes were prepared from 1.4 ml of this homogenate. The remaining homogenate and microsomes suspended in 0.1 M sodium phosphate buffer (pH 7.4) were stored at −20°C. ALA synthase activity was determined as in [9] with some modifications [8]. PBG synthase activity was measured as in [10] with the following modifications: The incubation mixture consisted of 0.1 ml homogenate and 0.4 ml 0.1 M sodium phosphate buffer containing 5 μmol ALA and 1.5 μmol reduced glutathione (pH 6.3). After incubation for 45 min the reaction was stopped with 0.5 ml 4% trichloroacetic acid–2.7% $HgCl_2$. An equal volume of modified Ehrlich's reagent was added to the supernatant after centrifugation and the A_{556} was measured after 15 min. When cultures or homogenates were treated with SA, non-enzymatic formation of a pyrrole from SA and ALA was observed. Total cellular heme concentration was determined by the oxalic acid–fluorimetric procedure as in [11]. Microsomal cytochrome P450 concentration was measured in microcuvettes according to [6] and protein concentrations were determined by the Lowry method [12].

3. RESULTS

In cultured chick embryo hepatocytes phenobarbital increased ALA synthase activity (2-fold) and cytochrome P450 concentration (2-fold), whereas PBG synthase activity remained unchanged. Addition of SA increased the phenobarbital-mediated induction of ALA synthase, strongly inhibited PBG synthase activity and decreased cytochrome P450 concentration. In control cultures, not exposed to phenobarbital, the effects of SA on heme synthesis and cytochrome P450 were similar, but not as pronounced as when induction causes enhanced intracellular heme requirement for the synthesis and assembly of cytochrome P450 hemoproteins (fig.1). The effect of SA on ALA synthase and on cytochrome P450 concentrations in monolayers exposed to phenobarbital was dose-dependent (fig.2). Furthermore, the phenobarbital-mediated increase in total cellular heme was also reduced by SA in a dose-dependent

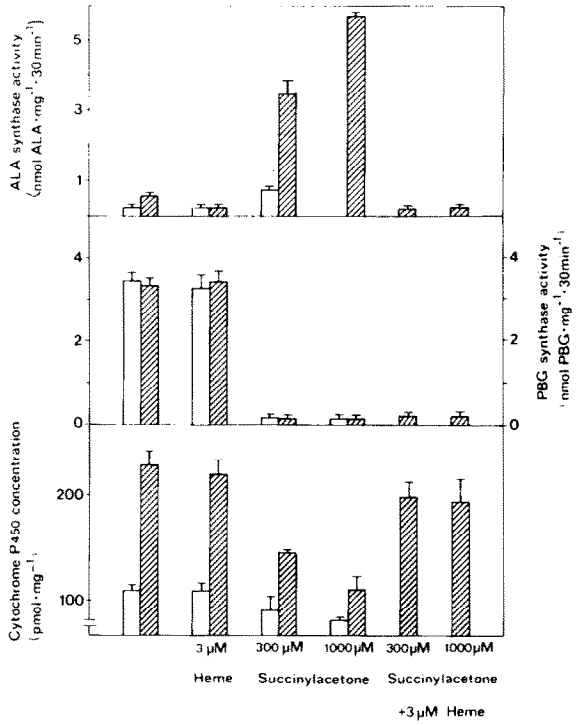

Fig.1. Effect of succinylacetone on ALA synthase and PBG synthase activities and cytochrome P450 concentration in hepatocyte culture. Hepatocyte monolayers were exposed to SA and/or phenobarbital (0.4 mM) for the final 20 h or 16 h of a 44 h culture period, respectively. Heme (3 μM) was added twice 18 h and 12 h prior to the preparation of homogenate and microsomes. Each bar represents the mean ± SE of 3–4 independent expt: (□) phenobarbital-treated cultures; (□) control (no phenobarbital treatment).

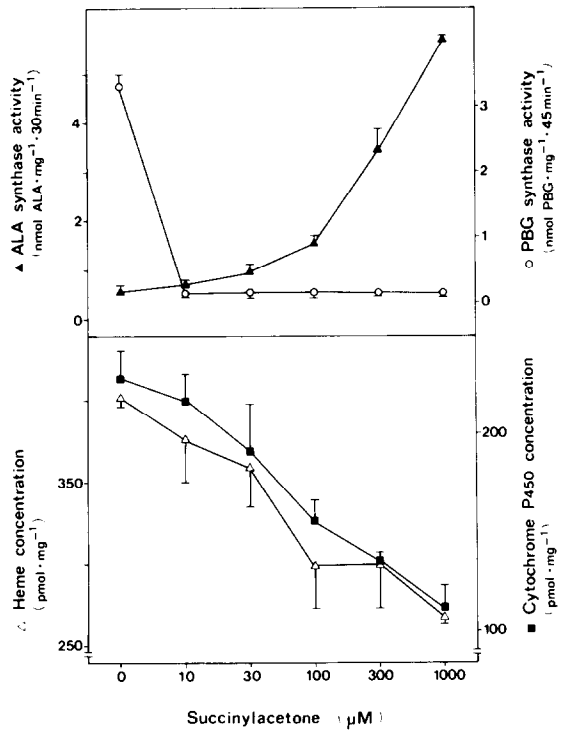

Fig.2. Dose–response of succinylacetone on ALA synthase and PBG synthase activities, cellular heme and cytochrome P450 concentration in hepatocyte cultures treated with phenobarbital. Hepatocytes were exposed to SA for the last 20 h of culture, and phenobarbital (0.4 mM) was added for the final 16 h. Each point represents the mean ± SE of 3–4 independent expt.

manner towards basal cellular heme concentrations (control content 299 ± 16 pmol heme/mg protein, n = 4), parallel to the impaired phenobarbital-mediated induction of cytochrome P450 by SA (fig.2).

The inhibition of heme synthesis at the level of PBG synthase by SA in hepatocyte culture could be overcome by the addition of exogenous heme (fig.1). Heme alone did not affect PBG synthase activity or cytochrome P450 concentration, but suppressed the phenobarbital-mediated induction of ALA synthase. However, the SA-mediated inhibition of cytochrome P450 induction by phenobarbital was almost completely reversed by adding exogenous heme to the medium. Moreover, heme entirely repressed the increase of ALA synthase activity by SA.

4. DISCUSSION

The present study in cultured chick embryo hepatocytes demonstrates that succinylacetone is an extremely potent inhibitor of hepatic heme synthesis at the level of PBG synthase. Moreover, our data suggest that impaired phenobarbital-mediated induction of cytochrome P450 and an enhanced induction of ALA synthase may both be explained by the depletion of intracellular heme, since these effects of SA were reversed by exogenous heme. These studies confirm and extend observations on the effect of SA in rats in vivo [2].

In hepatocytes, the second enzyme of heme biosynthesis, PBG synthase, which catalyses the conversion of two δ-aminolevulinate into porphobilinogen, is present in considerable excess in relation to the rate-limiting enzyme of this pathway. Therefore, PBG synthase activity has to be strongly inhibited to affect heme synthesis. At 10 μM SA, this enzyme activity was $>95\%$ inhibited, but cellular heme concentration and cytochrome P450 induction were only minimally altered. The enzyme activity apparently was further inhibited by increasing the concentration of SA in the culture medium as indicated by the dose-dependent enhanced induction of ALA synthase, the progressively impaired induction of cytochrome P450 and the loss of cellular heme. In contrast to ALA synthase, the activity of PBG synthase appears to be neither increased by phenobarbital nor inhibited by exogenous heme in cultured chick embryo hepatocytes.

Other inhibitors of hepatic PBG synthase activity, such as lead [13], levulinate, 3-amino-1,2,4-triazole, EDTA [2] or L-2-amino-4-methoxy-*trans*-3-butenoic acid [14] are either less potent or less specific than SA [2]. As with other inhibitors of heme synthesis [8], the effect of SA on ALA synthase was potentiated by simultaneous exposure to phenobarbital, which induces apocytochrome(s) P450 [7,8,15]. It is likely that this extensive induction of ALA synthase is caused by the combination of an extremely restricted cellular heme pool through SA-mediated inhibition of heme production and enhanced consumption of heme for the assembly of cytochrome P450 hemoproteins. This striking induction of ALA synthase has been observed with other inhibitors of heme synthesis given in combination with inducers of

cytochrome P450 [16,17].

Our study in cultured hepatocytes further indicates that SA does not inhibit synthesis of apocytochrome P450, for instance by known effects of heme on the initiation of synthesis of some other proteins [18], but blocks the assembly of the hemoprotein. The observation that the effect of SA on induction of cytochrome P450 was almost completely abolished by addition of heme to the medium suggests that exogenous heme does not only get access to the intracellular heme pool(s) regulating ALA synthase, but also is incorporated into newly synthesized apoprotein. 3-Amino-1,2,4-triazole, another inhibitor of PBG synthase, was found to impair synthesis of cytochrome P450 in rat liver; this inhibition was partially reversed by the addition of heme [19]. In murine erythroleukemia cells, the SA-mediated decrease of protein synthesis and cell growth was partially counteracted by the addition of heme to the medium [20,21].

The results of this experimental study suggest important clinical implications for patients with hereditary tyrosinemia:

(i) Drugs known to precipitate acute attacks in hepatic porphyria induce hepatic cytochrome(s) P450 and ALA synthase. As the effect of SA apparently imitates the genetic enzyme deficiencies of porphyria, clinical symptoms of patients with hereditary tyrosinemia are likely to be aggravated by the same drugs.

(ii) Accumulation of potentially neurotoxic ALA and impaired heme availability for cytochrome P450 may be influenced by the administration of exogenous heme, similar to the proposed mechanism of the treatment of patients with acute attacks of hepatic porphyria [22].

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