HYDROCORTISONE INDUCTION OF PHENYLALANINE HYDROXYLASE ISOZYMES IN CULTURED HEPATOMA CELLS

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SUMMARY: The hydrocortisone stimulation of phenylalanine hydroxylase activity in Reuber H4 hepatoma cells is shown to be associated with an alteration in phenylalanine hydroxylase isozyme composition. Three forms of phenylalanine hydroxylase were identified in H4 cells which have been treated with hydrocortisone; however, only one of these forms appears to be present prior to glucocorticoid treatment. The relative amounts, as well as the total amount, of the three forms and their chromatographic behavior on hydroxylapatite are nearly identical to the three phenylalanine hydroxylase isozymes found in adult rat liver. The hydroxylase isozyme composition in 2 day old rats is similar to that found in adult rats and in H4 cells treated with hydrocortisone.

Although little is known concerning the *in vivo* regulation of the hepatic enzyme phenylalanine hydroxylase, Koller *et al.* (1) found that hydrocortisone injections increased the phenylalanine hydroxylase activity 3-fold in adrenalectomized rats, and McGee *et al.* (2) reported that hydrocortisone increased the hydroxylase activity 2-fold, to nearly adult levels, in rats less than two weeks old. In cultured rat hepatoma cells, hydrocortisone has been shown to increase the phenylalanine hydroxylase specific activity 3- to 6-fold (3,4,5) to a specific activity comparable to that of adult rat liver.

Glucocorticoids have been shown to increase the rate of synthesis of several enzymes (see 6 for review), and to affect the isozyme pattern of at least one enzyme, tyrosine aminotransferase (7,8). Three forms of phenylalanine hydroxylase have been identified in adult rat liver (9,10). Tourian *et al.* (11) recently reported that one immunochemically identifiable phenylalanine hydroxylase isozyme was present in Reuber H4 hepatoma cells. We had made a similar observation using hydroxylapatite chromatography to separate the isozymes. We have found, in addition, that the phenylalanine hydroxylase isozyme in H4 cells has the same subunit molecular weight (5) and

is chromatographically indistinguishable from one of the three forms of phenylalanine hydroxylase found in adult rat liver (10). In this communication, we have investigated the effect of hydrocortisone stimulation on the phenylalanine hydroxylase isozyme complement present in H4 cells growing in culture.

METHODS

Reuber H4 hepatoma cells (12) were grown in monolayer culture using a modification (13) of medium S-77 as described (4). In the experiments presented, all cell cultures were near or at their maximum population density. H4 cell extract and rat liver extract were prepared as described (4). Phenylalanine hydroxylase isozymes were fractionated by a modification of the procedure of Barranger *et al.* (9); see Figure 1 for details. Recoveries of phenylalanine hydroxylase activity applied to the hydroxylapatite column of 90% have been obtained with rat liver extracts and extracts from hydrocortisone stimulated cell cultures and of 70% with normal H4 cultures.

In all fractionations, rat hemoglobin, prepared from rat erythrocytes, was mixed with the cell or rat liver extract prior to their application to the hydroxylapatite column. Under our conditions, this protein eluted immediately after the final phenylalanine hydroxylase isozyme, and served as a convenient internal standard for elution. Although not indicated on the figures, total protein distributed in a random fashion throughout the gradient. Phenylalanine hydroxylase activity was measured by fluorometric tyrosine determination (14), using the assay mixture described for our spectrophotometric assay (4).

RESULTS

Effect of Hydrocortisone on Phenylalanine Hydroxylase Isozymes in Reuber H4 Hepatoma Cells - To determine if hydrocortisone treatment altered the phenylalanine hydroxylase isozyme pattern in H4 cells, cell cultures were grown in the presence or absence (control) of 10^{-5} M hydrocortisone for 24 hr. At that time, cell extracts were prepared and fractionated on hydroxylapatite columns. Figure 1 shows the isozyme composition of control cultures and hydrocortisone treated cultures. Only one major form of phenylalanine hydroxylase is seen in control cells (Fig. 1A), in agreement with other reports (10,11). It has an elution position of 0.81, relative to rat hemoglobin, which is identical to that of isozyme I of rat liver (10). However, three phenylalanine hydroxylase isozymes could be separated in extracts from hydrocortisone treated cultures (Fig. 1B). These three isozymes eluted in the same positions, relative to the hemoglobin standard, as the 3 phenylalanine hydroxylase isozymes from adult rat liver: .81, .66 and .46 (Fig. 1B) compared to .80,

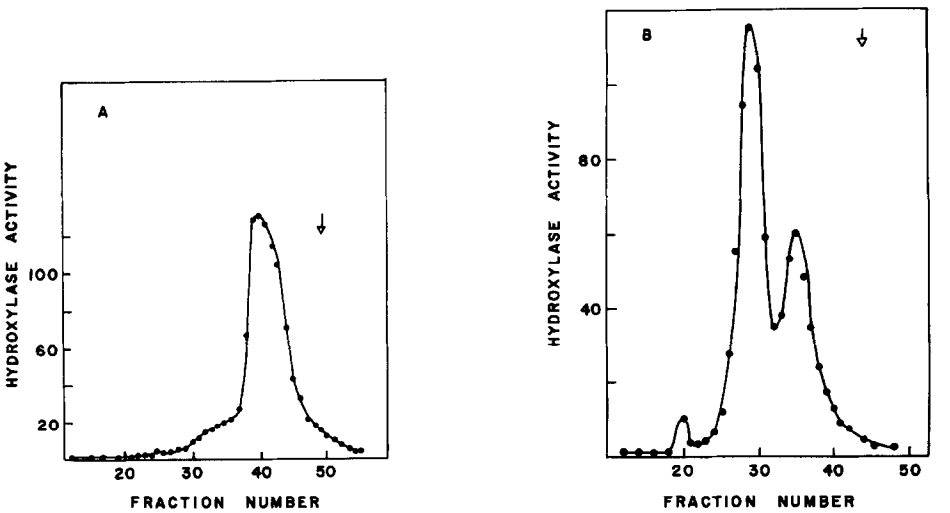

Figure 1: Hydroxylapatite chromatography of phenylalanine hydroxylase from A.) normal cultures and B.) cultures treated with $2x10^{-5}$ M hydrocortisone for 24 hours. In both cases, 9 confluent T-250 flasks were pooled and sonicated (4) in 0.7 ml 0.025 M potassium phosphate buffer (pH 6.8) containing 0.15 M KCl, 5 mM dithiothreitol, 20 mM phenylalanine and rat hemoglobin. The extracts were centrifuged at 40,000xg for 20 min. 0.6 ml of the clear supernatant was applied to a 0.5 x 20 cm column of hydroxylapatite, which had been equilibrated with the homogenization buffer. The enzyme was eluted with a 30 ml potassium phosphate linear gradient (0.025 M - 0.40 M), pH 6.8, containing 0.15 M KCl, 5 mM dithiothreitol and 20 mM phenylalanine. The flow rate was about 0.1 ml/min and 0.4±.05 ml fractions were collected. All procedures were performed at 0-4°. Ordinate of A: phenylalanine hydroxylase activity in nmoles tyrosine formed/300 µl/20 min. Ordinate of B: activity in nmoles tyrosine formed/ 150 µl/20 min. Abscissas: Number of fractions collected after initiating the gradient flow. Arrows indicate the position of the reference protein, rat hemoglobin, measured at 420 nm. Phenylalanine hydroxylase specific activity in the normal culture extract was 4.3 nmoles tyrosine/min/mg protein, and in the hydrocortisone treated was 16 nmoles tyrosine/min/mg protein.

.68 and .49 (10) for forms I, II and III, respectively. Barranger *et al.* (9) reported that the relative activity of isozymes I, II and III, in adult rat liver, was about 6:8:1, respectively, which we have confirmed. The same three isozymes from the hydrocortisone stimulated cultures (Fig. 1B) have relative activities of 6:9:0.2. Thus, except for the somewhat lower amount of the minor isozyme III, the hydrocortisone induced and adult rat liver phenylalanine hydroxylase isozyme complements appear the same.

The data in Figure 1 indicate that hydrocortisone had two effects on the

H4 cell cultures. It induced the expression of isozymes II and III, and it also caused about a 1.5-fold increase in the activity of isozyme I. (This was calculated from the 3.6-fold stimulation by hydrocortisone in overall enzyme activity, and the observed 6:9 ratio of activity of isozymes I and II in the stimulated cultures.)

Phenylalanine Hydroxylase Isozymes in 2 Day Old Rats - Liver phenylalanine hydroxylase specific activity has been reported to rise from nearly zero before birth to 20-30% of the adult rat liver value by 2 days after birth (2). The isoenzyme distribution for newborn rats was not known; although human fetal liver was reported to have only two phenylalanine hydroxylase isozymes, corresponding to forms I and III (9). After chromatography, the isozyme complement in livers from 2 day rats appears to be the same as that observed in hydrocortisone treated H4 cultures (Fig. 1B) and in adult rat livers (9,10). The three hydroxylase isozymes observed in the young rats chromatographed in the same position (.83, .70 and .48 relative to rat hemoglobin) as the three isozymes in adult rats, and the relative activity of these isozymes was approximately 6:7:0.3. It appears that even though the cultured hepatoma cells and the liver cells of young rats both exhibit a low phenylalanine hydroxylase specific activity which can be stimulated by hydrocortisone (2,4,5) to adult rat liver specific activity, the phenylalanine hydroxylase isozyme compositions in these two systems are quite different (Fig. 1A and Fig. 2). There also seems to be no obvious relationship between the human fetal isozyme expression and that of the newborn rats.

DISCUSSION

This is the first report to demonstrate that the phenylalanine hydroxylase isozyme composition of H4 cells is altered by hydrocortisone. Stimulation of the hydroxylase activity by hydrocortisone was shown to result in the production of two hydroxylase isozymes (II and III) which were not normally observed in H4 cells (Fig. 1), and to stimulate about 1.5-fold the activity of isozyme I, which is present in H4 cells in the absence of added hormone.

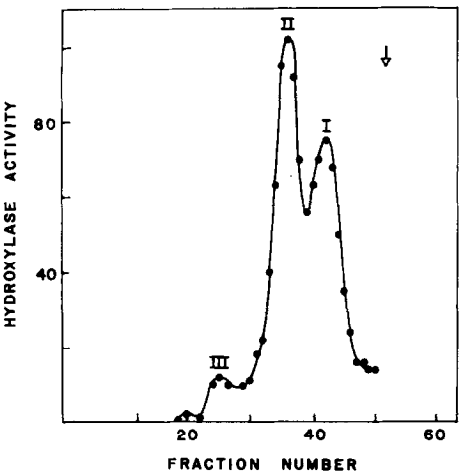

Figure 2: Chromatography of extracts from 2 day old rats. Extract was prepared by homogenizing livers from 2 day old Sprague-Dawley rats with a teflon homogenizer in 2 volumes of buffer containing 0.025 M potassium phosphate (pH 6.8), 0.15 M KCl, 5 mM dithiothreitol and 20 mM L-phenylalanine. The homogenate was centrifuged at 40,000xg for 20 min to yield the rat liver extract. All procedures are at 0-4°. 0.6 ml of extract was applied to a 0.5 x 20 cm hydroxylapatite column and eluted as in Figure 1. 200 µl aliquots were assayed for activity. Phenylalanine hydroxylase specific activity of the extract was 6.5 nmoles tyrosine formed/min/mg protein. Ordinate: phenylalanine hydroxylase activity in nmoles tyrosine formed/200 µl/20 min. Abscissa: number of fractions collected after starting the gradient flow.

The manner in which hydrocortisone induces the hydroxylase activity is not known; however, two lines of evidence indicate it does not simply activate an inactive form of the enzyme. McClure *et al.* (5) have shown that hydrocortisone stimulation of phenylalanine hydroxylase activity is accompanied by a proportionate increase in the rate of amino acid incorporation into H4 cell hydroxylase, and Haggerty *et al.* (3) have demonstrated that cycloheximide completely prevents the hydrocortisone induction of hydroxylase activity in these cells.

There is a striking similarity between our findings on the isozymes of phenylalanine hydroxylase and results from several groups on the isozymes of tyrosine aminotransferase (7,15,16,17). In that case, the isozymes appear to result from post-translational modifications of a parent protein (7,15,16), the chemical nature of which is still not clear (7,17,18).

It is not yet known whether phenylalanine hydroxylase isoenzymes are formed from subunits with different amino acid composition or from covalent modifications of one protein. In the absence of that information, two interpretations of our data are possible: 1) hydrocortisone induces the expression of at least one new hydroxylase subunit and also increases the rate of synthesis of the "constitutive" subunit; 2) hydrocortisone induces an increased overall rate of phenylalanine hydroxylase synthesis and also stimulates a protein modification system.

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