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CHARACTERIZATION OF VARIATIONS IN RABBIT HEPATIC PROGESTERONE 21-HYDROXYLASE ACTIVITY BY SERIAL BIOPSY

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SUMMARY: Earlier work has shown that the 21-hydroxylation of progesterone in the hepatic microsomal fraction of outbred New Zealand White rabbits varies over a 10-fold range. To determine whether the differences in 21-hydroxylase activity were due to a transient inductive effect, livers from a group of 28 rabbits were serially biopsied at least three times over a minimum period of two months. Both progesterone 21- and 16α-hydroxylase activities were determined in the post-8700g supernatant of homogenates prepared from these biopsy samples. A substantial variability in both the 21- and 16α-hydroxylase activity was observed for serial biopsy samples from individual rabbits. Each animal was found, however, to maintain relatively constant ratios of 21/16α-hydroxylase activity throughout the course of the study. Previous studies have indicated that the 21-hydroxylase activity does not correlate with the 16α-hydroxylase activity and that the 21-hydroxylase phenotype could be determined from the ratio of these activities. On the basis of this ratio, two groups of animals could be distinguished in the present study. Approximately 25% of the animals exhibited an elevated 21/16α-hydroxylase ratio (>1.5), the remainder were below this level. Furthermore, the expression of elevated levels of the 21-hydroxylase activity were found to be consistent within this subpopulation suggesting that a transient inductive effect is not responsible for the differences in 21-hydroxylase activity among populations of outbred New Zealand White rabbits. This study demonstrates the determination of the hepatic enzymatic phenotype while maintaining the animal for long periods of time and for subsequent investigations.

In normal subjects most deoxycorticosterone (DOC)[1] is synthesized in the endoplasmic reticulum of the zona fasciculata of the adrenal cortex by the hydroxylation of progesterone at carbon 21. Progesterone 21-hydroxylase activity has also been found in several other tissues,

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[1]Abbreviations used are: DOC, deoxycorticosterone; NZW, New Zealand White.

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but normally the contribution of these peripheral sites to the production of circulating DOC is minimal (1). During late pregnancy, however, when plasma levels of progesterone are markedly elevated, a large fraction of circulating DOC is derived from the extra-adrenal conversion of progesterone (1,2). Also, in the congenital adrenal hyperplasias (adrenal 21-hydroxylase deficiencies), approximately one-third of the production of DOC can take place in extra-adrenal tissues (3) contributing to the maintenance in some cases of low-normal to normal serum DOC values (4).

It has been demonstrated that a ten-fold variation in the fractional conversion of plasma progesterone to DOC exists in the human population under normal physiologic conditions (1). Similar differences are also apparent among NZW rabbits which exhibit a greater than ten-fold difference in hepatic progesterone 21-hydroxylase activity (5). This observation, made on single determinations for 23 rabbits, raises the question of whether such a difference constitutes a transient difference in regulation or reflects a heterogeneity in the expression of this enzyme in the rabbit population employed. To distinguish between these two possibilities, we measured the hepatic 21- hydroxylase activity of 28 rabbits by serial liver biopsies obtained over a period exceeding two months.

MATERIALS AND METHODS

A panel of 28 adult NZW rabbits, 10 males and 18 females, weighing 2.5 to 3.0 kg, were purchased from CAMM Research Laboratory Animals, New Jersey. The animals were kept in the same living quarters for the duration of the study and fed standard laboratory diet. At least three liver biopsies were taken from each animal, separated by intervals generally exceeding three weeks.

Approximately 20 mg of liver tissue were obtained each time by punch liver biopsy (TRU-CUT disposable needle, Travenol Lab Inc., Deerfield, Illinois) after anesthetization of the animal with sodium pentobarbital (22 mg/kg). Tissues were homogenized in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol (10 µl/mg tissue). The homogenate was then centrifuged at 8700 x g for 30 minutes and the supernatant fraction collected and diluted 1:10 for assay. Because of the small sample size, microsomes could not be isolated from the fraction in measurable quantities.

The procedure for measurement of progesterone 16α- and 21-hydroxylase activities has been reported (5). Briefly, the reaction mixture (1 ml) contained 50 μmol of potassium phosphate, pH 7.4, 50 μl of the 8700g supernatant and 10 nmol of [^{14}C]progesterone (Amersham; 56 mCi/mmol) added in 10 μl of methanol. After a preincubation of 3 minutes at 37°C, the reaction was initiated by the addition of 0.5 μmol of reduced nicotinamide adenine dinucleotide phosphate. Following an incubation of 5 minutes at 37°C, the reaction was terminated by extraction of substrate and products into 9 ml of chloroform. An aliquot of the organic phase was subsequently dried under a stream of nitrogen at 45°C. Progesterone and its metabolites were redissolved in 25 μl of ethyl acetate and applied to a thin layer chromatography plate coated with silica gel (Bakerflex IBF-2) by means of an Atlas semiautomatic applicator (Analytichem International). The plates were developed by sequential application of two solvent systems, isopropyl ether/acetone (80:20) and benzene/ethyl acetate/acetone (80:10:10). The portions of the silica gel plate that contained radioactive material, visualized by autoradiography, were removed for scintillation counting. The total recovery of progesterone and its metabolites was 85±10% (5). This procedure resolves 3 major metabolites of progesterone: 6β-; 16α-; and 21-hydroxyprogesterone. The rates of formation of these metabolites are linear with respect to protein concentration and incubation time. All results are expressed as the mean ± SD. The statistical significance between two measurements was determined using Student's t-test.

RESULTS

The hepatic 21- and 16α-hydroxylase activity of 28 NZW rabbits was determined at least 3 times at 3-week intervals. The activities were determined on post-8700g supernatants of liver biopsy homogenates and were expressed per milligram wet tissue weight. Figure 1 shows sequential measurements of 21- and 16α-hydroxylase activities for the 7 animals which exhibited 21-hydroxylase activity that exceeded 0.1 nmol/min/mg wet tissue. Substantial variability was found among the individual 21-hydroxylase activity measurements made in each animal. Yet, in each case, the variation in the 16α-hydroxylase activity paralleled that of the 21-hydroxylase.

Earlier results obtained for hepatic microsomal samples isolated from untreated adult NZW rabbits did not show a correlation in the activities of the 16α-hydroxylase and 21-hydroxylase (6) and the variation in 16α-hydroxylase activity in the earlier study was minor in comparison to that of the 21-hydroxylase activity. The parallel variation seen for the serial biopsy samples suggested that compositional heterogeneity due to the presence of fatty and connective

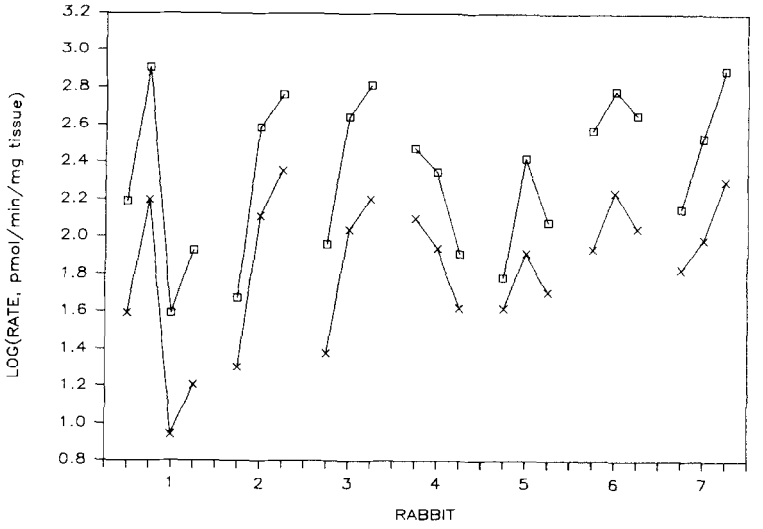

Figure 1 The progesterone 21- and 16α-hydroxylase activity of serial liver biopsy samples taken over a 2-month period from rabbits exhibiting elevated 21-hydroxylase activity. Each pair of lines denotes one of seven rabbits, with the upper line (□) depicting the 21- and the lower line (X) the 16α-hydroxylase activity. The variation of the 16α-hydroxylase activity among biopsy samples for individual rabbits was found to parallel that of the 21-hydroxylase activity in each case.

tissue, as well as contaminating material such as blood, likely to be present in biopsy samples, contributed to differences between samples for both activites. This assumption was supported by the observation of varying erythrocyte content in the pellets obtained by centrifugation. We therefore chose the 16α-hydroxylase as a reference enzyme. As shown in Figure 2, the variation in the ratio of 21- to 16α-hydroxylase activities is much less extensive for individual animals than that seen for either enzyme expressed in terms of wet weight of the biopsy sample. Moreover, the data in Figure 2 clearly distinguish two groups of animals having high (>1.5) and low ratios of 21- to 16α-hydroxylase activity. From previous results obtained for hepatic microsomes, we have determined that the ratio was 3.8 $\pm$ 1.6 for those animals exhibiting high rates of 21-hydroxylase activity and 0.5 $\pm$ 0.2 for those that were low. In the present study, the average ratio for the high group was 3.2 $\pm$ 1.2 and that for the low activity group was 0.8 $\pm$ 0.3. The similarity of the ratios determined in the present study with those obtained by

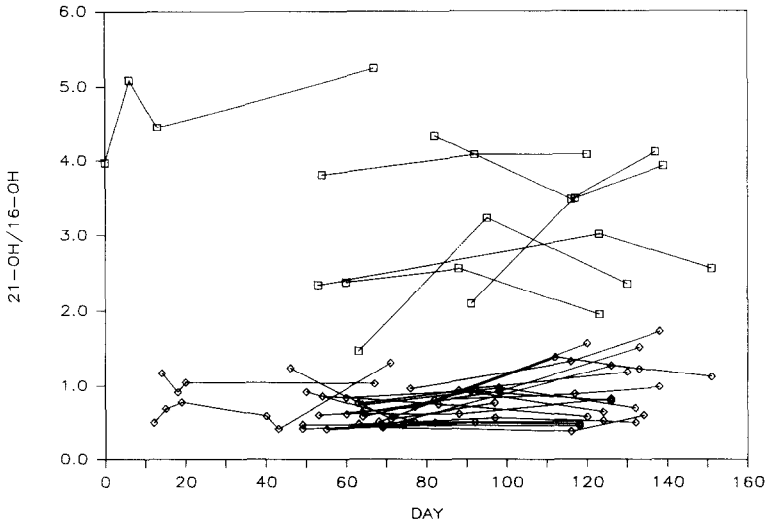

Figure 2 The ratio of 21- to 16α-hydroxylase activity determined for serial liver biopsy samples from 28 New Zealand White rabbits of either sex.

quantitation of microsomal levels further validate the claim that the 21- to 16α-hydroxylase ratio can be used to phenotype the rabbits.

The internal variation in levels within each of the two groups in the present study was computed as a coefficient of variation: the value of 15.2 ± 6.9, found in rabbits with high enzyme activity, is 40% smaller than the coefficient of variation calculated from the measurements in the animals with low levels of hepatic 21-hydroxylase activity, 25.1 ± 17.9. This difference is not statistically significant. The incidence of high 21-hydroxylase activity was approximately 25%, and was found to be independent of the sex of the animal. These results were consistent with earlier findings in the determination of 21-hydroxylase activity phenotype among rabbits from liver microsomal preparations (5).

DISCUSSION

Earlier work established that hepatic 21-hydroxylase activity varies extensively among NZW rabbits (5). Additional investigations indicated that this activity is largely catalyzed by a single P-450 enzyme, P-450 1 (7,8). The results of immunoquantitation indicate that the microsomal concentration of this enzyme is highly correlated to the

level of enzyme activity (9). In contrast, the 16α-hydroxylase activity is catalyzed by several P-450 enzymes with P-450 3b contributing more than 50% of the activity (10). The microsomal concentration of the latter enzyme is relatively constant as opposed to the variation seen for P-450 1. The differences in the expression of P-450 1 might reflect the induction of this P-450 by environmental or dietary factors giving rise to a transient increase in the concentration of the cytochrome and the 21-hydroxylase activity. Many of the hepatic forms of P-450 are known to be induced by treatment with foreign compounds (6). On the other hand, these differences in activity might reflect genetic differences regulating the expression of this enzyme. In support of the latter hypothesis, we have not observed the high 21-hydroxylase phenotype among several inbred strains of rabbit suggesting that this is a heritable trait among these strains (unpublished).

The results of the present study suggest that hereditary factors predominate over environmental factors in the expression of 21-hydroxylase activity. The serial biopsy samples show a relatively constant level of 21-hydroxylase activity over a period of over two months with respect to that of 16α-hydroxylase, and show in addition that the ratio of 21- to 16α-hydroxylase activity distinguishes two phenotypes. We attribute the variation seen in both activities expressed in terms of wet weight of the biopsy sample to differences in the composition of the sample. On the basis of the ratio of 21- to 16α-hydroxylase activity, the animals in this study can be grouped as exhibiting either high or low 21-hydroxylase activity, the high 21-hydroxylase activity phenotype occurring in about 25% of the animals. This result is consistent with data obtained for single-measurement determinations of 21-hydroxylase activity from rabbit hepatic microsomes (5).

It should be emphasized that this methodology can usefully be extended to include further tests for the inducibility of hepatic P-450

isozymes in the same animals for which it allows a phenotype to be established. We are currently investigating the mode of inheritance of the phenotype established by breeding rabbits expressing a high level of 21-hydroxylase activity, as determined herein, with inbred strains which do not exhibit elevated levels of this activity.

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