Vol. 84, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1978 Pages 706-712

# IS STEROID-16α-HYDROXYLASE SUPPORTED BY MORE THAN ONE MONOOXYGENASE?

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Received June 20, 1978

Summary

Steroid-16α-hydroxylase activities have been measured in normal and induced rat livers using four different substrates. The male/female activity ratio as well as the induction factor vary with the substrate indicating that steroid-16α-hydroxylase activity is a heterogenous enzyme. Experiments using specific inhibitors led to the conclusion that steroid-16α-hydroxylase is supported by at least two cytochrome P-450 forms, different from the cytochrome P-448.

## Introduction

Steroid hormones are essentially deactivated in the liver. Several enzymatic systems are involved in their biotransformation which after dehydrogenation, reduction, hydroxylation and conjugation ultimately result in the production of more polar and therefore more hydrosoluble metabolites. The oxidation reactions involved in this process are catalyzed by the microsomal cytochrome P-450 mediated monooxygenases.

This multienzymatic system is known to be responsible for the metabolism of a wide variety of exogenous and endogenous lipophilic chemicals such as: drugs, polycyclic hydrocarbons, insecticides, biogenic amines, fatty acids, cholesterol... (1). Several studies (2,3) have demonstrated that this enzymatic complex is heterogenous. The catalytic entity, namely cytochrome P-450, exists in multiple forms which may be characterized by their substrate specificity, their sensitivity to the action of specific inhibitors as well as their physico-chemical properties (spectral characteristics, molecular weight... (4,5,6).

From the point of view of the endogenous substrates, steroids provide the biochemist with a unique tool to study the monooxygenase heterogeneity: (a) a given steroid can be hydroxylated on a number of positions and (b) a variety of steroids can be hydroxylated on the same position.

This paper implements the advantages of our recently described assays for studying the 16α-hydroxylation of four steroids, i.e. testosterone, progesterone, dehydroepiandrosterone and pregnenolone.

Important variations in the respective oxidation rates of the four substrates and in the *in vitro* action of specific inhibitors were found

0006-291X/78/0843-0706$01.00/0

when comparing the enzymatic activity of samples prepared from differently treated male or female rats. These observations led us to postulate the existence of more than one steroid-16α-hydroxylase in the rat liver.

## Methods

Male and female Sprague-Dawley rats (150-200 g) were used. Sodium phenobarbital was given intraperitoneally in a daily dose of 50 mg/kg body weight for four days. Methylcholanthrene and 16α-cyanopregnenolone, dissolved in corn oil, were given intraperitoneally in a dose of 20 mg/kg for three days.

Liver homogenates and microsome isolation were realized as described in an earlier publication (7). Protein concentrations were determined according to Lowry *et al.* (8) with bovine serum albumin being used as a standard.

The measurement of the 16α-hydroxylase activity was performed by incubating specifically 16-tritiated steroids (pregnenolone, progesterone, testosterone and dehydroepiandrosterone) and by counting the tritium released in the medium. These methods have been described in detail elsewhere (7,9). All assays were run in triplicate. The coefficient of variation was estimated from a series of 10 identical determinations to be 3%. Aryl hydrocarbon hydroxylase activity was measured by the fluorimetric method according to Nebert and Gelboin (10) and recorded on an Aminco Bowman spectrofluorimeter. Microsomal cytochrome P-450 concentration was measured according to Omura and Sato (11).

## Results and Discussion

Table 1 shows the steroid-16α-hydroxylase activity measured with different substrates in control, phenobarbital (PB), methylcholanthrene (MC) and 16α-cyanopregnenolone (PCN) treated rat liver microsomes. In a given group of rats, the level of steroid-16α-hydroxylase activity varies significantly from one substrate to another. For instance, in the control rats, the testosterone-16α-hydroxylase activity is only 57 pmol/min/mg protein while the DHEA 16α-hydroxylase activity is about 10 times higher (574 pmol/min/mg protein). In addition, regardless of the substrate used, important differences were found when comparing the enzymatic activities of male and female animals. Higher activities were always found in the male rats; the male/female ratio varying from 1.1 up to 12.

PB induces the four 16α-hydroxylases at least by a factor of 2 in all animals, but again, important variations were observed in the degree of induction of the different hydroxylases. DHEA and pregnenolone-16α-hydroxylase activity were only induced in the male rat by a factor of 2 while progesterone-16α-hydroxylase was induced more than 8 fold in the male and female animals. MC reduces the enzymatic activity in male rats, whereas no significant changes were produced in females. Finally, PCN was a good inducer of all 16α-hydroxylases, but was more efficient in the female as compared to the male animals.

Table 1

Liver 16α-hydroxylase activity measured with different substrates (Pregnenolone, progesterone, testosterone, dehydroepiandrosterone) after induction of male and female rats. The enzymatic activities (mean + S.D.; n = 5) are expressed in pmol x $min^{-1}$ x mg $protein^{-1}$. Cytochrome P 450 concentrations are expressed in nmol x mg $protein^{-1}$.

| PRETREATMENT | SEX | TESTOSTERONE -16α-HOASE | PROGESTERONE -16α-HOASE | DHEA -16α-HOASE | PREGNENOLONE -16α-HOASE | CYT. P 450 |
|---|---|---|---|---|---|---|
| NONE | ♂ | 57 ± 24 | 118 ± 34 | 574 ± 210 | 78 ± 18 | 0.59 |
| | ♀ | 4.7 ± 1.7 | 96 ± 14 | 80 ± 30 | 7.7 ± 1.9 | 0.43 |
| PHENOBARBITAL | ♂ | 179 ± 53 | 850 ± 241 | 974 ± 434 | 169 ± 52 | 1.79 |
| | ♀ | 16 ± 5 | 555 ± 105 | 175 ± 77 | 64 ± 19 | 0.73 |
| METHYLCHOLANTHRENE | ♂ | 16 ± 5 | 48 ± 11 | 470 ± 173 | 38 ± 6 | 0.73 |
| | ♀ | 4.5 ± 2.1 | 93 ± 24 | 67 ± 27 | 5.1 ± 2.4 | 0.61 |
| 16-CYANOPREGNENOLONE | ♂ | 134 ± 12 | 193 ± 17 | 1324 ± 96 | 142 ± 21 | 0.74 |
| | ♀ | 22 ± 8 | 173 ± 58 | 190 ± 27 | 103 ± 18 | 0.75 |

From the results shown, the variation in inducibility and in the male-female activity ratio are not directly correlated to a modification of cytochrome P-450 concentration in the microsomal preparation.

In order to further characterize the biochemical properties of the different 16α-hydroxylases, we have investigated the *in vitro* effects of specific monooxygenase inhibitors : α-naphtoflavone (ANF), metyrapone (MTR), and tetrahydrofurane (THF) (11, 12). The action of these compounds on aryl hydrocarbon hydroxylase was also measured and used as a reference.

ANF, a preferential inhibitor of the cytochrome P-448 mediated monooxygenases, did not modify the steroid-16α-hydroxylase activity in the control or induced groups of rats (Fig. 1). The results were less homogenous with the female derived enzymes, ANF slightly enhancing the testosterone and the pregnenolone-16α-hydroxylase in the PB treated group and significantly depressed the progesterone-16α-hydroxylase in the MC treated animals (Table 2). When the ANF concentration was raised further in the incubation mixture, the behavior of the steroid-16α-hydroxylase activities was parallel to that of PB induced AHH, i.e. a cytochrome P-450 supported monooxygenase. Figure 1 illustrates the phenomenon in the case of a progesterone-16α-hydroxylase, similar results (data not shown) were obtained with the other substrates.

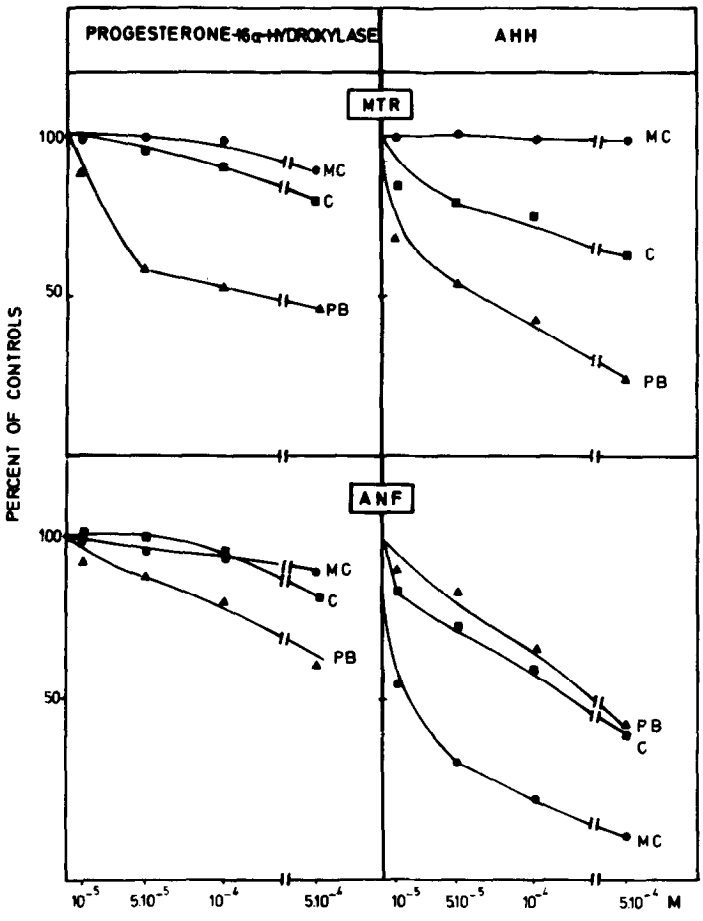


Figure 1: Inhibition of progesterone-16α-hydroxylase and AHH by metyrapone and α-naphtoflavone.

Table 2

Inhibition of steroid-16α-hydroxylase and AHH by α-naphtoflavone ($5.10^{-5}$ M). Results are expressed in percent of the corresponding non inhibited control.

| SEX | PRETREATMENT | TESTOSTERONE -16α-HOASE | PROGESTERONE -16α-HOASE | DHEA -16α-HOASE | PREGNENOLONE -16α-HOASE | AHH |
|---|---|---|---|---|---|---|
| MALE | NONE | 83 | 92 | 88 | 93 | 50 |
| | PHENOBARBITAL | 82 | 80 | 90 | 81 | 75 |
| | METHYLCHOLANTHRENE | 84 | 87 | 91 | 84 | 30 |
| FEMALE | NONE | 89 | 81 | 80 | 130 | 72 |
| | PHENOBARBITAL | 130 | 90 | 86 | 150 | 91 |
| | METHYLCHOLANTHRENE | 76 | 53 | 70 | 90 | 50 |

Table 3

Inhibition of steroid-16α-hydroxylase and AHH by metyrapone ($5.10^{-5}$ M). Results are expressed in percent of the corresponding non inhibited control.

| SEX | PRETREATMENT | TESTOSTERONE -16α-HOASE | PROGESTERONE -16α-HOASE | DHEA -16α-HOASE | PREGNENOLONE -16α-HOASE | AHH |
|---|---|---|---|---|---|---|
| MALE | NONE | 94 | 92 | 91 | 55 | 86 |
| | PHENOBARBITAL | 68 | 57 | 68 | 37 | 54 |
| | METHYLCHOLANTHRENE | 94 | 100 | 88 | 73 | 100 |
| FEMALE | NONE | 81 | 69 | 96 | 67 | 85 |
| | PHENOBARBITAL | 63 | 42 | 80 | 46 | 65 |
| | METHYLCHOLANTHRENE | 94 | 84 | 100 | 63 | 100 |

Table 3 shows that MTR did not modify the different steroid-16α-hydroxylases in both the control and the MC treated animals, but very significantly inhibited the PB induced enzymes, regardless of the steroid used as substrate. The pattern of MTR action was, in fact, very similar to that observed with the AHH. This is further demonstrated in Figure 1 where the action of MTR on the progesterone-16α-hydroxylase was studied in function of the drug concentration.

Finally, the effect of THF, a preferential inhibitor of ethanol inducible female enzyme (4) was tested on the steroid-16α-hydroxylases. No significant modification (data not shown) of the control or induced enzyme activities could be demonstrated.

## Discussion

The cytochrome P-450 dependent monooxygenases have been mainly studied using synthetic chemicals as substrates. The steroids, as many other endogenous compounds, are also actively metabolized by these enzymes, but have only been utilized occasionally in order to characterize the biochemical properties of this system. This situation is obviously due to the complexity of the steroid metabolic pathways (13,14,15) which render the precise determination of a specific monooxygenase activity tedious and inaccurate.

The development of new assays easy to perform now allow the study of specific steroid hydroxylases. We have previously defined the general biochemical properties of the steroid-16α-hydroxylase using 4 different steroids as substrates: testosterone, progesterone, dehydroepiandrosterone and pregnenolone (7,9). In this paper, we reported the results of a study on the influence of sex and chemical inducers on the steroid-16α-hydroxylase acti-

vities. Basically, we observed: (a) The four different substrates are not hydroxylated with the same efficiency; (b) the male/female ratio of the enzymatic activity varies from one substrate to another and (c) the administration of inducers affects differently the oxidation of the four steroids. These data are very hard to explain except if one postulates the existence of more than one steroid-16α-hydroxylase. Such a situation has been postulated for different drug metabolizing monooxygenases, such as the ethylmorphine demethylase (16,17) or the aryl hydrocarbon hydroxylase (18). In the case of the steroids, it is documented that different monooxygenases are involved in the hydroxylation of different carbons (19,20). Concerning the enzyme hydroxylating a given carbon on different steroids, a possibility of a heterogenous system active on cholesterol, testosterone and biliary acids (15) has been raised for the 7α-hydroxylase.

Our results thus suggest that the 16α-hydroxylation of the steroids can be supported by more than one monooxygenase. The particular sensitivity of both the male and female PB-induced 16α-hydroxylase to metyrapone indicated the existence of a new monooxygenase (or a conformational change) capable of hydroxylating the steroids on the 16α-position. As this new form of enzyme is not present in the control preparations, it will thus be necessary in order to explain also the male/female differences, to postulate the existence of at least three different forms of cytochrome P-450 which could support the 16α-hydroxylation of the steroids. As ANF is not able to inhibit the 16α-hydroxylase activity, we can assume that cytochrome P-448 cannot support this reaction. Moreover, the negative action of THF rules out the participation of the ethanol inducible cytochrome P-450.

Important differences exist in the *in vitro* inhibition of each of the 4 steroid-16α-hydroxylations by the three other substrates (manuscript in preparation) and supports the hypothesis of three different enzymes. Experiments are presently in progress in order to solubilize the enzymatic complex and to further characterize the cytochrome P-450(s) involved in the steroid-16α-hydroxylation.

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