16β-HYDROXYLATION OF 4-ANDROSTENE-3,17-DIONE IN PHENOBARBITAL-PRETREATED RAT LIVER MICROSOMES

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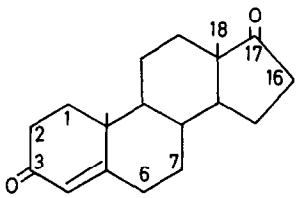
Received 8 February 1980

1. Introduction

There are several reports on the hydroxylation of 4-androstene-3,17-dione (androstenedione) at the 2β-, 6β-, 6α-, 7α-, 16α- and 18-positions by adult male rat liver microsomes [1–5], and Einarsson et al. [6] suggested the presence of weak 16β-hydroxylase activity in male Long–Evans strain rat liver microsomes, but so far, there is no definite proof of the presence of androstenedione 16β-hydroxylase activity in male rat liver microsomes. During studies on the metabolism of androstenedione in male rat liver microsomes, we observed that a new hydroxylation product became the predominant one with phenobarbital-pretreated rat liver microsomes, but it was only a minor product when control male rat liver microsomes were used for incubation. This paper reports the selective induction of androstenedione 16β-hydroxylase activity in adult male rat liver microsomes by phenobarbital pretreatment.



Androstenedione

2. Materials and methods

17β-Hydroxy-4-androsten-3-one (testosterone) and androstenedione were commercial products, 16α-hydroxyandrostenedione was the gift of Dr. Tamaoki and 7α-hydroxyandrostenedione and 16β, 17α-dihydroxy-4-androstene-3-one were the gift of Dr. Komeno, 6α-hydroxyandrostenedione and 6β-hydroxyandrostenedione were the gift of Dr. Hayakawa.

Mass spectra were measured with a Hitachi RMU-6 instrument and IR spectra with a Jasco DS-403E instrument in a KBr tablet.

80 mg/kg body weight of sodium phenobarbital in saline solution were given intraperitoneally to 3 male rats of Wistar strain (8 weeks old) for 3 days and 3 control rats were given saline only. The animals were sacrificed on the 4th day. Liver homogenate was prepared in 4 vol. of 0.25 M sucrose solution containing 0.05 M Tris–HCl buffer (pH 7.4) with a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 5000 × *g* for 10 min and the supernatant was further centrifuged at 105 000 × *g* for 60 min. The precipitate was suspended in the homogenizing medium, 1 ml/g tissue; an equal volume of glycerol was added to the suspension which was then stored at −20°C. The hydroxylase activities of microsome preparations stored at −20°C in 50% glycerol suspension did not decrease at least for one month.

Small-scale incubations were performed in 50 ml flasks containing 750 μg of androstenedione in 0.1 ml of methanol, 0.2–0.4 ml of rat liver microsomal preparation, 1.5 mg of NADP, 9 mg of glucose-6-phosphate, 3 units of glucose-6-phosphate dehydrogenase, 0.3 ml of 0.5 M $MgSO_4$ solution and the final volume was adjusted to 9 ml with the 0.05 M Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose solution. Incubation was at 37°C for 15 min with constant shaking. The incubation mixture was extracted twice with 2 vol. of dichloromethane, the combined organic

layers were dried over sodium sulfate, evaporated under reduced pressure and the residue was dissolved in a small amount of dichloromethane, spotted onto a silica gel thin layer plate (Merck precoated silica gel 60F-254) and chromatographed in solvent mixture A: dichloromethane:acetone (4:1). The plate was developed twice to 15 cm from the origin.

To prepare UV scanograms, 0.1 vol. of the extract of a small scale incubation mixture was chromatographed on a silica gel thin layer plate which was then scanned over the range 240–360 nm with a Shimadzu CS-900 dual wavelength thin layer chromatogram scanner.

3. Results

3.1. *Comparison of control and phenobarbital induced rat liver microsomal androstenedione hydroxylase activity*

The most pronounced differences in the incubation products of androstenedione between control and phenobarbital pretreated rat liver microsomes were in bands IV and V (fig.1). Band IV was almost absent in the incubation products of androstenedione with the control rat liver microsomes, but it was the most abundant UV absorbing product with the phenobarbital-pretreated rat liver microsomes. The chromatographic mobilities of bands I, III and V corresponded to 7α-hydroxyandrostenedione, 6β-hydroxyandrostenedione and 16α-hydroxyandrostenedione, respectively.

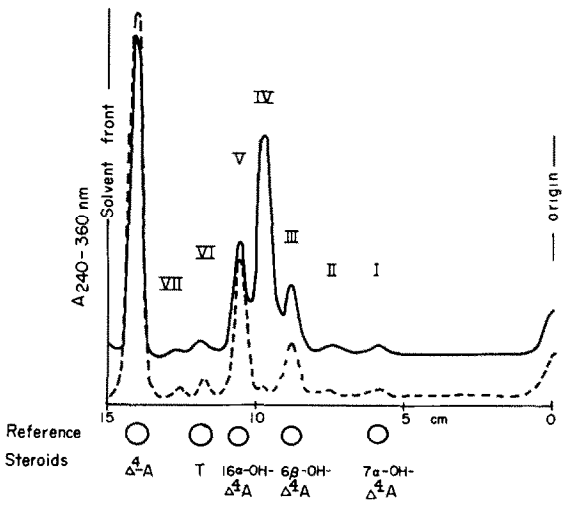


Fig.1. UV scanogram of a thin layer chromatograph of incubation products of androstenedione with 0.4 ml of control (– – –) or 0.3 ml of phenobarbital (——)-pretreated rat liver microsomes. *Abbreviations:* Δ^4-A, androstenedione; T, testosterone.

3.2. *Isolation of compound IV and V*

A 100 times larger scale incubation of androstenedione with phenobarbital-pretreated rat liver microsomes was performed to prepare compound IV, and the extract was chromatographed on silica gel thin layer plates in solvent system A. The plates were developed twice to obtain better separation of each band. The strongest UV absorbing band (band IV) and the UV absorbing band just above it (band V) were scratched off and eluted with a mixture of dichloromethane:acetone (2:1). The eluates of band IV and V were rechromatographed twice in solvent system B; dichloromethane:ethanol (50:1), then each band was eluted. The eluate of band V (compound V) was identified as 16α-hydroxyandrostenedione by comparison of its IR and mass spectra with those of an authentic sample.

3.3. *Properties of compound IV*

Since the compound IV (eluate of band IV) gave a positive blue tetrazolium reaction, it should contain a ketol group. Its mass spectrum gave $M^+ = 302$, and its fragmentation pattern resembled that of 16α-hydroxyandrostenedione. Furthermore, compound IV was rather unstable, a large part of it being converted into a slightly less polar compound, IVa, when a methanol solution of it was left at room temperature for several days. Compound IVa also gave a positive blue tetrazolium reaction and its mass spectrum was almost identical to that of compound IV. It has already shown that the steroids having the 17-oxo-16β-ol structure, such as 3β,16β-dihydroxy-5-androsten-17-one or 16β-hydroxyestratrien-17-one, are easily isomerized to the more stable 16-oxo-17β-ol structure [7–9], and the isomerized products (16-oxo-17β-ol steroids) show chromatographic mobilities that were slightly higher than the parent compounds [8]. From the above features, compounds IV and IVa were considered to be 16β-hydroxyandrostenedione and 16-oxo-testosterone. To confirm this postulate, compounds IV and IVa were reduced with a small amount of $NaBH_4$ in methanol at 0°C. The major reduction product of both compounds IV and IVa were a little more polar than compound IV

in solvent system A. Since an acetonide was easily formed by the addition of *p*-toluene sulfonic acid to acetone solution of reduced compounds IV and IVa, the reduction product should contain a *cis vic* diol, and it was identified as 16β-hydroxytestosterone from the IR spectrum. Furthermore compound IV gave a small amount of another UV absorbing band after reduction with $NaBH_4$, which showed similar thin layer chromatographic mobility to 16β,17α-dihydroxy-4-androsten-3-one in solvent mixture C; benzene:acetone (4:1). Compound IVa also gave a small amount of another reduction product corresponding to 16α-hydroxytestosterone (16α,17β-dihydroxy-4-androsten-3-one) in solvent mixture C. From these results, compound IV was identified as 16β-hydroxyandrostenedione and compound IVa as 16-oxo-testosterone.

4. Discussion

Several studies have reported the inducing effect of phenobarbital on rat liver microsomal steroid hydroxylase activities, but there are no reports about the induction of 16β-hydroxylase activity in rat liver microsomes by phenobarbital pretreatment. However, it is shown in this report that the most abundant hydroxylation product of androstenedione is 16β-hydroxyandrostenedione in phenobarbital pretreated rat liver microsomes. Therefore when discussing the inducing effect of phenobarbital treatment on androstenedione hydroxylase activities in male adult rat liver microsomes, it would be erroneous if examination of 16β-hydroxylase activity was omitted, since it is selectively induced in rat liver microsomes by phenobarbital pretreatment.

Previously Conney and Schneidman [11] reported that the production of an unknown hydroxylated metabolite after incubation of androstenedione with phenylbutazone-pretreated immature rat liver microsomes. The unknown product should be 16β-hydroxyandrostenedione, because the major hydroxylase activity on androstenedione became the 16β-hydroxylase after phenylbutazone pretreatment of immature rats (unpublished observation).

The inducing effect of phenobarbital has been believed to be rather broad and nonspecific, but it has sharp specificity if limited to the activities of androstenedione hydroxylases.

Recently, Hrycay et al. [12] reported the increased production of 16-oxo-testosterone from androstenedione in male rat liver microsomes after phenobarbital pretreatment, and they presumed that it would be the rearranged product of 16α- or 16β-hydroxyandrostenedione, but they could not isolate 16β-hydroxyandrostenedione. As stated earlier, 16β-hydroxyandrostenedione is rather labile, and if it is left on a silica gel thin layer plate for long periods, all of it will be converted into 16-oxo-testosterone. Hrycay et al. used an autoradiogram technique to detect the radioactive metabolites on thin layer chromatograms, and silica gel thin layer plates were left for 6 days in contact with X-ray films before characterization of each metabolite. It will be enough to complete the isomerization of 16β-hydroxyandrostenedione to 16-oxo testosterone. Furthermore the thin layer chromatographic mobility of 16β-hydroxyandrostenedione is a little less than that of 16α-hydroxyandrostenedione, but 16-oxo-testosterone is inseparable from 16α-hydroxyandrostenedione in the solvent system chloroform:ethyl acetate (4:1), therefore the band they were characterizing as 16-oxo-testosterone was probably 16β-hydroxyandrostenedione.

We are very much indebted to the mentioned colleagues for the generous gifts of various standard steroids.

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