Vol. 93, No. 4, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1980 Pages 1014-1019

INDUCTION OF TESTOSTERONE 16β-HYDROXYLASE IN RAT LIVER MICROSOMES BY PHENOBARBITAL PRETREATMENT

Yutaka Nakamura and Seiko Ueda

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka, 553, Japan

Received March 5, 1980

Summary Oxidation products of testosterone in control rat liver microsomes were 16α-, 2α-, 6β-, 7α-hydroxytestosterone and 4-androstene-3,17-dione, but no 2β-hydroxytestosterone was detected. Increased testosterone 16β-hydroxylase activity and 4-androstene-3,17-dione production were found upon incubation of testosterone with phenobarbital-pretreated rat liver microsomes.

Major testosterone hydroxylase activities in male rat liver microsomes have been considered to occur at the 2β-, 6β-, 7α-, and 16α-positions (1-7), but recently the presence of 2α-hydroxylase activity was also reported (8-10). Induction of testosterone hydroxylase activity in adult male rat liver microsomes by phenobarbital treatment has been studied (4,11), but testosterone 16β-hydroxylase activity has not been reported. Recently, selective induction of androstenedione 16β-hydroxylase activity in male rat liver microsomes by phenobarbital treatment has been observed (12). Since androstenedione 16β-hydroxylase was expected to also attack testosterone, 16β-hydroxylation of testosterone in rat liver microsomes was studied.

MATERIALS AND METHODS

2α-Hydroxytestosterone was prepared from 2α-acetoxy-17β-propoxy-4-androsten-3-one by hydrolysis with $KHCO_3$ in boiling methanol (13) and purified by thin layer chromatography in solvent mixture A. Mp 163-165°C, $(\alpha)_D^{24}$ $CHCl_3$: +121.0 ± 3.2, MS m/e 304 (M^+), CD: (methanol) [θ] (λ_{nm}) -8,240 (320). 2β-Hydroxytestosterone was prepared from 2β-acetoxy-17β-propoxy-4-androsten-3-one by incubation with male rat liver microsomes at 37°C for 4 hours. By this treatment, ester bonds were hydrolyzed by microsomal esterase and further conversion of 2β-hydroxytestosterone into 2-oxotestosterone was minimized. Crude 2β-hydroxytestosterone was purified by thin layer chromatography in solvent mixture A. Mp 161-163°C, $(\alpha)_D^{24}$ $CHCl_3$: -139.6 ± 3.5, MS m/e 304 (M^+), CD: (methanol) [θ] (λ_{nm}) +5,270 (317). 16α-Hydroxytestosterone was prepared from 16α-hydroxyandrostenedione by reduction with $NaBH_4$ in methanol at 0°C.

0006-291X/80/0801014-06$01.00/0

7α-Hydroxytestosterone was the gift of Dr. Komeno, 2α-acetoxy-17β-propoxy-4-androsten-3-one and 2β-acetoxy-17β-propoxy-4-androsten-3-one were gifts of Dr. Kubota, and 16α-hydroxyandrostenedione was the gift of Dr. Tamaoki. 16β-Hydroxy-androstenedione was prepared by incubation of androstenedione with phenobarbital-treated rat liver microsomes and purified by thin layer chromatography in solvent systems A and B, mp 119-121°C. The detailed preparation method of this compound will be presented in another report.

Precoated thin layer plates (silica gel 60 F-254, Merck) were used for the separation of steroids. The following thin layer chromatographic solvent systems were used: A, dichloromethane:acetone (4:1); B, dichloromethane:ethanol (20:1); C, cyclohexane:ethylacetate (1:2); D, benzene:acetone (2:1).

Adult male rats of the Wistar strain 8-10 weeks old were intraperitoneally given saline or sodium phenobarbital (80 mg/kg) in saline once a day for three days, and killed on the fourth day. Their livers were homogenized using a Teflon pestled homogenizer with four volumes of 0.25 M sucrose containing 0.05 M tris-HCl buffer solution (sucrose buffer). The homogenate was centrifuged at 9,000 x g for 10 min, and the supernatant was further centrifuged at 105,000 x g for 60 min. The precipitate was suspended in the sucrose buffer and centrifuged again (105,000 x g for 60 min). The final precipitate was resuspended in the sucrose buffer and made up to 1 gm liver tissue/ml, then an equal volume of glycerol was added, and this was mixed well and stored at -20°C.

Small-scale incubation: Rat liver microsomes (control, 0.4 ml or phenobarbital-treated, 0.3 ml) were added to a mixture of 3 mg NADP, 9 mg glucose-6-phosphate, 3 units of glucose-6-phosphate dehydrogenase, and 150 μmoles of $MgSO_4$, and the final volume was adjusted to 9 ml with the sucrose buffer. Incubation was started by the addition of testosterone (750 μg in 0.1 ml of methanol solution). After 15 min incubation at 37°C, the mixture was extracted with two volumes of dichloromethane twice, then the combined extracts were washed with one-tenth volume of water, dried over sodium sulfate and evaporated. The residue was dissolved in 0.5 ml of ethyl acetate and 50 μl of this was spotted on a thin layer plate, then reference steroids were spotted on the left side of the plate, and the plate was developed in solvent system A twice. The uv scanogram of the chromatographed plates were prepared using a Shimadzu CS-900 dual wave length scanner at the 240-360 nm setting.

Mass spectra were measured with a Hitachi OMU-6 mass spectrometer and ir spectra with a Jasco DS-403E infra red spectrometer as micro KBr tablets.

RESULTS

As shown in Fig. 1, the major uv-absorbing products after incubation of testosterone with control male rat liver microsomes were bands I, II, IV and VII. On the other hand, two new metabolites, bands III and V, were detected in the incubation products of testosterone with phenobarbital-treated rat liver microsomes.

To characterize and isolate the metabolites, large-scale incubation was performed (100-fold the usual incubation). The extracts were chromatographed in solvent system A

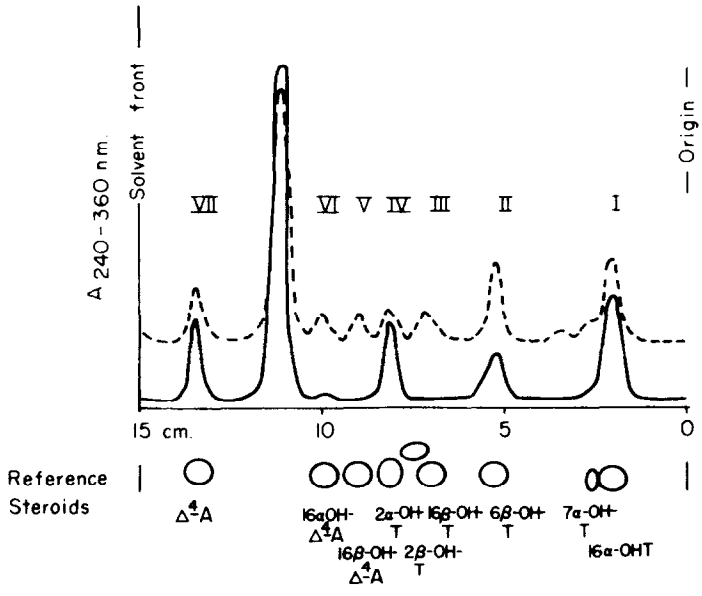

Fig. 1. Uv scanogram of the incubation products of testosterone with control and phenobarbital-treated rat liver microsomes. Control rat liver microsomes (0.4 ml) —— or phenobarbital-treated rat liver microsomes (0.3 ml) --- were incubated under standard conditions, and a one-tenth volume of each extract was spotted on a thin layer plate and chromatographed. The plate was scanned at the wavelength setting of 240-360 nm with a Shimadzu CS-900 dual wavelength chromatogram scanner. Δ^4-A, androstenedione; T, testosterone.

three times, then the uv absorbing bands I-VII were scratched off and eluted with dichloromethane:acetone (2:1).

1) Characterization of the testosterone metabolites in control rat liver microsomes.

Band I: The extract of this band was separated into two uv-absorbing bands corresponding to 16α-hydroxytestosterone (Ia) and 7α-hydroxytestosterone (Ib) after thin layer chromatography in solvent system D. Ia was the major component of band I, and Ib was a trace constituent. Further characterization of Ia was performed by the analysis of ir and mass spectra, and it was identified as 16α-hydroxytestosterone. Ib was identified as 7α-hydroxytestosterone by comparison of its thin layer chromatographic mobility in solvent mixtures B and E and its mass spectrum with those of the authentic steroid.

Band II: This band was separated into two uv-absorbing bands IIa and IIb after thin layer chromatography in solvent system C. The thin layer chromatographic

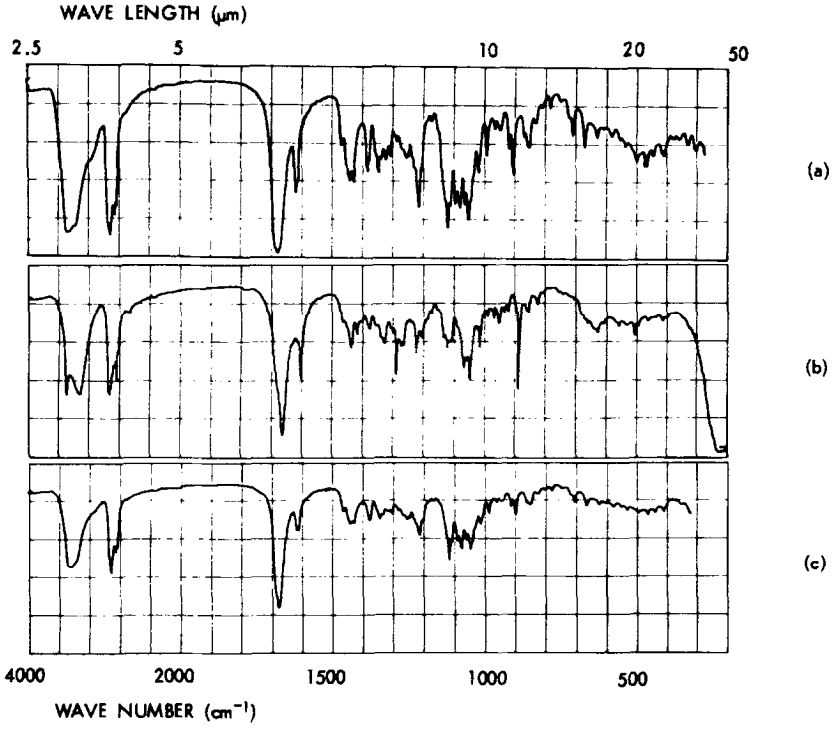

Fig. 2. Ir spectra of 2α-hydroxytestosterone (a), 2β-hydroxytestosterone (b) and compound IV (c).

mobility of the more polar band IIa was similar to that of 7α-hydroxyandrostenedione, but no further study was performed. Since the less polar band IIb showed the absorption maximum at 235 nm in methanol, and its sulfuric acid chromogen was similar to that reported for 6β-hydroxytestosterone (14), IIb was tentatively identified as 6β-hydroxytestosterone.

Band IV: The eluate of band IV was separated into IVa (the more polar band) and IVb (the less polar band) by solvent system C. The thin layer chromatographic mobility of band IVb was identical to that of 2α-hydroxytestosterone, but differed from that of 2β-hydroxytestosterone in three solvent systems (B, C, D). The ir spectra of 2α-hydroxytestosterone and IVb agreed well, but differed from 2α-hydroxytestosterone (Fig. 2). From these criteria, IVb was identified as 2α-hydroxytestosterone. IVa showed similar thin layer chromatographic mobility to that of 6β-hydroxyandrostenedione, but was not studied further.

Band VII: The thin layer chromatographic mobility of this band was the same as that of androstenedione in three different solvent systems (A, B, C). After purification by thin layer chromatography in solvent system C, VII was identified as androstenedione from its ir and mass spectra.

2) Characterization of testosterone metabolites in phenobarbital-treated rat liver microsomes.

Band V: The eluate of this band showed identical thin layer chromatographic mobility with that of 16β-hydroxyandrostenedione in solvent systems A, B, and C. When it was reduced with $NaBH_4$ in methanol at 0°C, it gave a uv-absorbing material which had similar chromatographic mobility to that of band III. The mass spectrum of band III gave M^+: 304 and both III and reduced V gave acetonide when a small amount of p-toluene sulfonic acid was added to their acetone solution. These features suggested the presence of vic cis diol. Since testosterone has the 17β-hydroxyl group, another hydroxyl group on III and reduced V should be at the 16β-position. From above results, III was identified as 16β-hydroxytestosterone and V as 16β-hydroxyandrostenedione.

DISCUSSION

The major hydroxylation products of testosterone in adult male rat liver microsomes were 16α-, 2α-, and 6β-hydroxytestosterones. In addition, a small amount of 7α-hydroxytestosterone and 16α-hydroxyandrostenedione was also produced. Since rather large quantities of androstenedione were produced from testosterone, 16α-hydroxyandrostenedione should be produced from androstenedione rather than from testosterone. This result differs from the previous report of Gustafsson et al. (15) which stated that 16α-hydroxyandrostenedione was not produced after incubation of testosterone with male rat liver microsomes.

Thus far studies on testosterone hydroxylases and the effect of phenobarbital pretreatment on rat liver microsomal steroid hydroxylase activity have been performed

neglecting studies of 2α- and 16β-hydroxylase activities, but 2α-hydroxylase is actually a major testosterone hydroxylase in male rat liver microsomes and 16β-hydroxylase also becomes a major one after phenobarbital treatment of the microsomes. Thus, studies of these hydroxylases activities should not be omitted from discussions on the inductive effect of phenobarbital treatment on testosterone hydroxylases activity in rat liver microsomes.

Several studies reported the presence of 2β-hydroxytestosterone after incubation of testosterone with male rat liver microsomes (2,7) or the perfusion of testosterone through male rat liver (15), but no detectable 2β-hydroxylase activity was found in adult male rat liver microsomes. Thus, it seems reasonable to conclude that the true hydroxylase activity of testosterone in normal adult male rat liver microsomes is due to 2α-hydroxylase and not to 2β-hydroxylase.

REFERENCES

1. Conney, A. H., and Klutch, A. (1963) J. Biol. Chem. 238, 1611-1617.
2. Lisboa, B. P., Gustafsson, J. Å., and Sjövall, J. (1968) Europ. J. Biochem. 4, 496-505.
3. Jacobson, M., and Kuntzman, R. (1969) Steroids 13, 327-341.
4. Levin, W., Alvares, A., Jacobson, M., and Kuntzman, R. (1969) Biochem. Pharmacol. 18, 883-889.
5. Welch, R. M., Levin, W., and Conney, A. H. (1967) J. Pharmacol. Exp. Ther. 155, 167-173.
6. Abraham, R., und Staudinger, Hj. (1966) Z. Physiol. Chem. 346, 198-207.
7. Shiverick, K. T., and Neims, A. H. (1979) Drug Metab. Dispos. 7, 290-295.
8. Wenzel, M., und Lemm, U. (1976) Z. Physiol. Chem. 357, 1145-1151.
9. Lemm, U., und Wenzel, M. (1978) Z. Physiol. Chem. 359, 81-88.
10. Homma, S., Iwamura, S., Yugi, S., Iizuka, K., Abe, T., and Kanbegawa, A. (1976) Proceedings of Eighth Symposium on Drug Metabolism and Action, pp. 91-94.
11. Kuntzman, R., Levin, W., Jacobson, M., and Conney, A. H. (1968) Life Sci. 7, 215-224.
12. Nakamura, Y., and Ueda, S. (1976) Seikagaku 48, 934.
13. Sondheimer, F., Kaufmann, St., Romo, J., Martinez, H., and Rosenkranz, G. (1953) J. Am. Chem. Soc. 75, 4712-4715.
14. Schriefers, H., Cremer, W. and Otto, M. (1967) Z. Physiol. Chem. 348, 183-193.
15. Gustafsson, J. Å., and Lisboa, B. P. (1970) Acta Endocrinol. 65, 84-94.