J. steroid Biochem. Vol. 23, No. 2, pp. 239–241, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

SHORT COMMUNICATION

THE METABOLISM OF ESTRADIOL 17-SULFATE BY PHEOCHROMOCYTOMA TISSUE

ITSUO YOSHIZAWA*, KAZUHIRO WATANABE*, SHINJI KUROSAWA† and SHOICHI NAKAGAWA†
*Hokkaido Institute of Pharmaceutical Sciences, 7-1, Katsuraoka-cho, Otaru, Hokkaido, 047-02, and
†Second Department of Medicine, Hokkaido University School of Medicine, Kita-ku, Sapporo, Hokkaido, 060, Japan

(*Received* 9 *October* 1984)

Summary—The metabolism of estradiol 17-sulfate by subcellular localization enzymes of pheochromocytoma tissue obtained from a 41-year old female was investigated. In any incubations under the presence of NADH and NADPH, metabolites hydroxylated at the C-2, C-4, C-6β, C-7α and C-7β positions were produced. These hydroxylations are considered to occur without cleavage of the sulfate group. The 2-hydroxylation at the substrate concentration of 100 μM by mitochondria, microsomes and cytosol fractions occurred at rates of 141, 222 and 167 pmol/mg protein/30 min, respectively; the corresponding rates for the 4-hydroxylation were 24, 40 and 38 pmol/mg protein/30 min. Mitochondrial 2- and 4-hydroxylations were enhanced by addition of calcium ion (Ca^{2+}) into the incubation medium.

INTRODUCTION

Pheochromocytoma is a tumor of the adrenal medulla, and has a characteristic that the chromaffin cells of its tissue secrete abnormal amounts of catecholamine. The mechanism of catecholamine secretion by this tumor, however, is not fully understood and the lack of information is due mainly to the tumor's rarity. Commonly, this disease is associated with a wide range of symptoms, and thus its diagnosis is generally very difficult [1].

Since the tumor cells originate and grow in the adrenal medulla, it was predicted from early times that steroid metabolism in adrenal cortex might be influenced [2]. Carballeria and Venning demonstrated that C_{21}-steroid precursors underwent some metabolism [3, 4]. Acevedo and Beering reported that estradiol was metabolized by this tumor tissue to the 2-hydroxylated products [5]. These results indicate that pheochromocytoma tissue has a capacity of steroid metabolism, although this tissue belongs to adrenal medulla.

Recently, we demonstrated 2- [6] and 4-hydroxylation [7] of estradiol 17-sulfate (I) by rat liver microsomes. This aromatic hydroxylation was also observed in brain, kidney, lung and heart [8]. Based on these results and also on those of Acevedo and Beering [5], we investigated whether pheochromocytoma tissue has a capacity of aromatic hydroxylation against I.

EXPERIMENTAL

A case report

The patient was a 41-year old Japanese female who exhibited fatigue, hypersweating and palpitation. She was said to have been hypertensive for several years and underwent a total hysterectomy because of myoma uterii. In her family history, no relatives had endocrinological disorders. On admission, a physical examination of her showed a blood pressure of 104/70 mmHg, pulse 90 and palor. Otherwise she was unremarkable. Laboratory values found for anterior pituitary, thyroid, parathyroid, pancreatic endocrine and adrenocortical function were normal. But urinary excretion of vanillylmandelic acid (VMA) ranged high levels (10–31 mg/day; normal: < 10 mg/day [9]), of adrenalin (445–521 μg/day; normal: 5–25 μg/day [9]), of noradrenalin (605–625 μg/day; normal: 20–50 μg/day [9]). Hypertensive state plasma concentration of adrenalin and noradrenalin were very high, 107–1180 pg/ml and 219–2430 pg/ml, respectively. Computalized axial tomography of the abdomen showed a right adrenal tumor shaped about 5 cm in diameter. Before the operation she was treated with labetarol (α,β-adrenergic blocker) 750 mg and prazosin (α-adrenergic blocker) 9 mg t.i.d., respectively. The tumor tissue, obtained in successful adrenalectomy, weighed 30 g and was immediately contributed to the following experiments. Histopathological finding showed a typical pheochromocytoma derived in adrenal medulla.

Materials

Free and conjugated steroids were prepared in this laboratory according to the known methods [10]. NADH and NADPH were purchased from Oriental Yeast, Inc. (Osaka, Japan). Sep-Pak C_{18} cartridges and Column guards were obtained from Waters, Ltd (Milford, MA, U.S.A.) and Millipore Co. (Bedford, MA, U.S.A.), respectively. All other reagents and solvents were obtained commercially.

High-Performance Liquid Chromatography (HPLC)

HPLC and preparative HPLC were carried out by the same machine under the same conditions as described in the previous paper [11]. The following solvents were used as mobile phases:

System A: a mixture of 0.5% $NH_4H_2PO_4$ (pH 3.0) and tetrahydrofuran (80:20, v/v), System B: a mixture of 0.5% $NH_4H_2PO_4$ (pH 3.0) and methanol (55:45, v/v), System C: a mixture of 0.5% $NH_4H_2PO_4$ (pH 3.0) and methanol (60:40, v/v).

Fractionation of cell components

A portion of the surgical specimen was carefully cleaned of all possible fragments in an ice-cold glass vessel. All the subsequent procedures were carried out at 0–4°C. The tissue was weighed and homogenized with 4-fold volume of an ice-cold 50 mM Tris–HCl buffer solution (pH 7.4) by a Teflon-glass Potter–Elvejhem homogenizer to give 20% (w/v) homogenate. Fractionation of the cell components was carried out on this homogenate by the same method of Hogeboom [12] except using buffer solution of 50 mM Tris–HCl (pH 7.4).

Determination of protein

Determination of protein contents in each fraction was carried out by the method of Lowry *et al.*[13] using bovine serum albumin as reference. The protein amounts of each fraction obtained from 1 g of tumor tissue were 28–30, 2–2.5, 7–8.5 and 17–20 mg for homogenates, mitochondria, microsomes and soluble fraction, respectively.

Incubation

Incubations were carried out under the following conditions. Ice-cold reaction vessels contained protein (0.5 ml), NADH (1 mM), NADPH (1 mM), KCl (90 mM), EDTA (0.1 mM) and the substrate (100 μM). Incubations using mitochondria were carried out in the presence or absence of $CaCl_2$ (11 mM). The mixtures were diluted with 50 mM Tris–HCl buffer solution (pH 7.4) to 3.0 ml as a final volume, and were incubated at 37°C under aerobic conditions for 30 min. The reaction was terminated by heating the incubation vessels in boiling water for 1 min, followed by addition of ascorbic acid (5 mg) as antioxidant and of a known amount of 2-hydroxyestradiol-3-methyl ether 17-sulfate as an internal standard (1.0–10 μg), then the mixture was diluted with 10 ml of water. Control experiments were performed using boiled microsomes (100°C for 1 min) by the same procedure as described above. The reaction mixtures were centrifuged at 1500 *g* for 20 min, and the precipitates suspended in water and again centrifuged. The combined supernatants were passed through Sep-pak C_{18} cartridges. After washing with 2 ml of water, the cartridges were eluted with methanol (4.0 ml) and the eluates evaporated under a nitrogen stream to give the residues, which were dissolved in 100 μl of methanol. These solutions were subjected to HPLC using system A as a mobile phase. At the same time, some of the incubation mixtures were added by 4-nitroestrone as an internal standard (about 2–5 μg), and the mixtures were extracted continuously with peroxide-free ether containing ascorbic acid (50 mg in 50 ml) for 18 h. The ether layer was washed with water, dried over anhydrous Na_2SO_4, and subjected to HPLC using system B.

Separation and solvolysis of steroid conjugates

The combined incubation mixture of three experiments (microsomes) was treated by the same method as described above, followed by the separation by preparative HPLC using system C. Four separated fractions corresponding to peaks 1, 2, 3 and 4 (Fig. 1) were then solvolyzed in acidic media [14]. After being added by 4-nitroestrone (about 5 μg), each solvolyzed product was employed for assignment of the products by HPLC using system B. Free steroids used for identification with each product were estradiol and its monohydroxylated derivatives; 2-, 4-, 6α-, 6β-, 7α-, 17β, 15α- and 16α-hydroxyestradiol.

RESULTS AND DISCUSSION

Studies on the metabolism of estradiol 17-sulfate (**I**) by pheochromocytoma tissue were undertaken. In the previous paper on the same substrate by rat liver microsomes, the incubations were carried out in the presence of NADPH-generating system [6, 7, 8, 11]. In the present experiments, however, incubations were done using NADH and NADPH, since it was obscure which coenzyme is required for this metabolism.

Regarding the formation of the products, almost the same tendencies were observed in any subcellular fractions. Ether extracts of all incubation mixtures contained negligible amounts of steroids, indicating that neither solvolysis of the substrate and product(s) nor resulfulyzation of the hydrolyzed metabolites could have taken place during the incubation. In contrast to the ether layer, over 90% of the substrate accompanied with its minor products remained in the aqueous layer in all experiments. These results indicate that hydroxylation of **I** by pheochromocytoma tissue occurred without removal of the sulfate group.

Figure 1 shows a chromatographic separation of authentic steroids (a), and of microsomal products (b), where four kinds of peak (1, 2, 3 and 4) are observed. Peaks 1, 2 and 3 are coincidental with authentic conjugates, estradiol 17-sulfate (**I**), 2-hydroxyestradiol 17-sulfate (**II**) and 4-hydroxyestradiol 17-sulfate (**III**), respectively. Peak 4 looks like a mixture of at least three kinds of products, the complete separation of which was unsuccessful.

The structure of the products was confirmed by the separation of these peaks using preparative HPLC, followed by their solvolysis. By HPLC of the solvolyzed products, only 2- and 4-hydroxyestradiol were detected in the fractions corresponding to peaks 2 and 3, respectively. On the other hand, 6β-, 7α- and 7β-hydroxyestradiol were detected in the solvolyzed products obtained from fraction corresponding to peak 4. Thus, it became evident that **I** was hydroxylated by pheochromocytoma tissue not only at C-2 and C-4 positions, but also at 6β, 7α and 7β positions. Similar results were also obtained by HPLC on the aqueous products of the homogenates and other subcellular fractions. No further investigation of the products in peak 4 was undertaken.

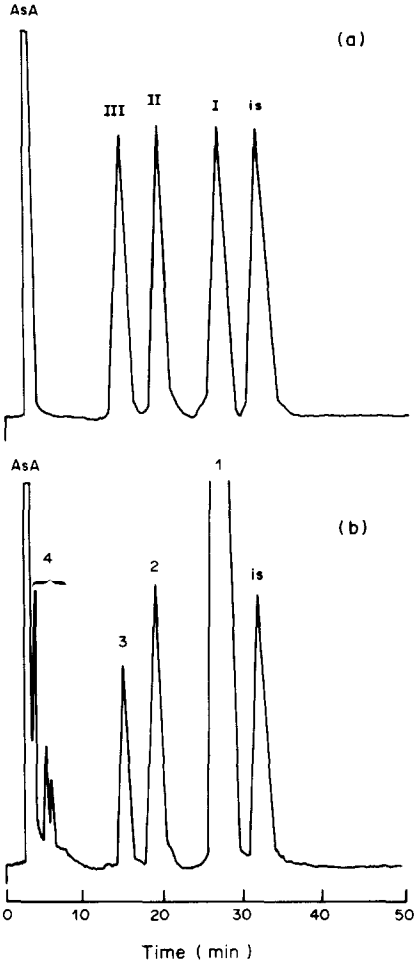

Fig. 1. High-performance liquid chromatograms of (a) authentic steroids and (b) incubation products of estradiol 17-sulfate by microsomes of pheochromocytoma tissue. **I**. estradiol 17-sulfate, **II**. 2-hydroxyestradiol 17-sulfate, **III**. 4-hydroxyestradiol 17-sulfate, **is**. 2-hydroxyestradiol-3-methyl ether 17-sulfate, AsA. ascorbic acid.

Table 1. Effect of calcium ion (Ca^{2+}) on 2- and 4-hydroxylation of estradiol 17-sulfate by mitochondrial fraction in pheochromocytoma tissue

Product	Yield (%)	
	$CaCl_2$ (−)	$CaCl_2$ (+)
2-OH-Product	0.09	0.19
4-OH-Product	0.04	0.07

Incubations were carried out in Tris–HCl buffer (pH 7.4, 50 mM) in the presence of NADH, NADPH, KCl and EDTA at the substrate concentration of 100 μM at 37°C for 60 min. The concentration of Ca^{2+} added was 11 mM. Results are mean values of duplicate experiments.

Table 2. Comparison of the enzyme activities at subcellular levels of estradiol 17-sulfate 2- and 4-hydroxylase in pheochromocytoma tissue

Fraction	Enzyme activity (pmol/mg protein/30 min)	
	2-Hydroxylase	4-Hydroxylase
Homogenate	60	17
Mitochondria	141	24
Microsomes	222	40
Cytosol	167	38

Incubations were carried out in Tris–HCl buffer (pH 7.4, 50 mM) in the presence of NADH, NADPH, KCl and EDTA at the substrate concentration of 100 μM at 37°C for 30 min. Ca^{2+} (11 mM) was added to the medium in the case of mitochondria. Results are mean values of duplicate experiments.

According to the recent report by Fishman *et al.*[15], catechol estrogen can be formed nonenzymatically. The catechol formation in the present experiments, however, is considered to be enzymatic, because no detectable amounts of the catechols was produced in the control experiments using boiled enzymes.

Peron *et al.*[16] reported that the *in vitro* hydroxylation of some adrenocortical steroids by rat liver mitochondria was enhanced under the presence of Ca^{2+}. Table 1 indicates the influence of Ca^{2+} upon the mitochondrial 2- and 4-hydroxylation of **I**: by addition of 11 mM of Ca^{2+}, hydroxylations at C-2 and C-4 increased by over 110 and 80%, respectively. This result makes us consider that the enhancement of the hydroxylation might be caused by an increase of not only the permeability of exogenous coenzymes [16], but also of the substrates, into the mitochondrial organella.

Because of the lack of sufficient tumor tissue, we could not investigate thoroughly the kinetics on the catechol formation from **I**. Incubations were, therefore, inevitably carried out at the substrate concentration of 100 μM as the close value to the apparent K_m values for microsomal estradiol 17-sulfate 2- and 4-hydroxylases [14]. Table 2 indicates that the activities of each fraction are essentially at almost the same level and that pheochromocytoma tissue has a fairly high 2- and 4-hydroxylase activity for **I**.

Comparison of the results by Acevedo and Beering[4] and ourselves seem to indicate a big difference in estradiol metabolism by pheochromocytoma tissue between free and 17-sulfated estradiol. The metabolism of free estradiol is essentially the C-2 oxidation, whereas that of the 17-sulfate is rather random hydroxylation. These results are unique, because the random hydroxylation of steroids is suppressed to become regiospecific by converting the free steroid substrates to their sulfoconjugates [17].

The results obtained by Acevedo and Beering[4] and also ourselves, made us expect that the patients with pheochromocytoma tumor might have high plasma or urinary catechol estrogen concentrations. Measurement of these catechol estrogens in body fluids might be useful for clinical diagnosis of this disease.

REFERENCES

1. Itoh T. and Ohmori K.: Biosynthesis and storage of catecholamines in pheochromocytoma and neuroblastoma cells. *J. Lab. clin. Med.* **81** (1973) 887–896.
2. Davignon J., Tremblay G. and Genest J.: Failure to isolate cortisol from a pheochromocytoma. *J. clin. Endocr. Metab.* **20** (1960) 1515–1520.
3. Carballeira A. and Venning E. H.: Conversion of steroids by chromaffin tissue. I. Studies with a pheochromocytoma. *Steroids* **4** (1964) 329–350.
4. Carballeria A. and Fishman L. M.: Steroid enzyme activities in extraadrenal pheochromocytomas. *J. clin. Endocr. Metab.* **54** (1982) 619–624.
5. Acevedo H. F. and Beering S. C.: The metabolism of 4-^{14}C-estradiol-17β by pheochromocytoma tissue. *Steroids* **6** (1965) 531–541.
6. Watanabe K. and Yoshizawa I.: Clinical analysis on steroids. XX. Estradiol 17β-sulfate as a substrate for 2-hydroxylation enzyme of rat liver microsomes. *J. pharmac. Dyn.* **5** (1982) 340–347.
7. Watanabe K., Kimura R. and Yoshizawa I.: Clinical analysis on steroids. XXIX. Evidence for 4-hydroxylation of estradiol 17-sulfate by rat liver microsomes. *Chem. pharmac. Bull.* **32** (1984) 2745–2751.
8. Watanabe K., Kimura R., Kameyama M. and Yoshizawa I.: Studies on the metabolism of estrogen conjugates by high-performance liquid chromatography. *J. pharmac. Dyn.* **7** (1984) s-20.
9. Feidman J. M., Blalock J. A., Zern R. T. and Welis S. A.: The relationship between enzyme activity and the catecholamine content and secretion of pheochromocytomas. *J. clin. Endocr. Metab.* **49** (1979) 445–451.
10. Watanabe K. and Yoshizawa I.: Clinical analysis on steroids. XXII. Preparation and high-performance liquid chromatographic determination of guaiacol estrogen 17β-conjugates; the enzymatic *O*-methylation products of 2-hydroxyestradiol 17β-conjugates. *Chem. pharmac. Bull.* **30** (1982) 3231–3238.
11. Watanabe K. and Yoshizawa I.: Clinical analysis on steroids. XXI. Assay of oestradiol 17-sulfate 4-hydroxylase activity by high-performance liquid chromatography with electrochemical detection. *J. Chromat.* **337** (1985) 114–120.
12. Hogeboom G. H.: *Methods in Enzymology* (Edited by S. P. Colowick and N. O. Kaplan). Academic Press, New York, Vol. 1 (1965) pp. 16–19.
13. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193** (1951) 265–275.
14. Watanabe K. and Yoshizawa I.: Clinical analysis on steroids. XXV. Assay of estradiol 17-sulfate 2-hydroxylase activity by high-performance liquid chromatography. *J. Chromat.* **277** (1983) 71–77.
15. Fishman J. and Norton B.: Specific and nonspecific components in the oxidative metabolism of estradiol by the male rat brain *in vitro*. *J. steroid Biochem.* **19** (1983) 219–228.
16. Peron F. G., Guerra F. and McCarthy J. L.: Further studies on the effect of calcium ions and corticosteroidogenesis. II. Adrenal mitochondrial swelling by calcium ions. *Biochim. biophys. Acta* **110** (1965) 277–289.
17. Gustafsson J.-Å. and Ingelman-Sundberg M.: Regulation and substrate specificity of a steroid sulfate-specific hydroxylase system in female rat liver microsomes. *J. biol. Chem.* **250** (1975) 3451–3458.