J. Steroid Biochem. Molec. Biol. Vol. 38, No. 4, pp. 533–535, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

SHORT COMMUNICATION

MITOCHONDRIAL *P*-450 ACTIVITIES IN ALDOSTERONOMA TISSUES

HIDEAKI TAKASAKI, ISAMU MIYAMORI,* KUNIO NAGAI, RYOYU TAKEDA, HIDEO MOCHIZUKI[1] and MASAYUKI KATAGIRI[1]

Second Department of Internal Medicine, School of Medicine, Kanazawa University and [1]Department of Chemistry, Faculty of Science, Kanazawa University, Kanazawa 920, Japan

(Received 8 January 1990)

Summary—Adrenal *P*-450 activities were measured by an *in vitro* reconstitution system from tissues obtained from human aldosteronomata, and the results compared with those of the normal adrenal tissues from patients with Grawitz's tumor. The $P\text{-}450_{11\beta}$ activity was significantly increased in adenoma tissue (55.6 ± 5.3 vs 9.0 ± 6.2 nmol corticosterone/mg of protein/min in the control tissues, $P < 0.01$). $P\text{-}450_{scc}$ activity in adrenal adenomata was 13.4 ± 2.0 nmol pregnenolone/mg of protein/min, significantly higher than control ($P < 0.05$). The present results suggest that increased mitochondrial $P\text{-}450_{11\beta}$ activities may be characteristic of aldosterone-producing adenomata.

INTRODUCTION

The steroid monooxygenase system is responsible for the biosynthesis of steroid hormones in the adrenal gland. Cholesterol side-chain cleavage (scc) is exclusively carried out by cytochrome $P\text{-}450_{scc}$. The bovine adrenocortical cytochrome $P\text{-}450_{11\beta}$ catalyzes hydroxylation of the steroids at the 11, 18 and 19 positions [1], and both *P*-450-linked enzymes are present in the mitochondrial intima. We are interested how these two types of mitochondrial *P*-450 enzymes function in aldosterone producing adenoma, where aldosterone and 18-hydroxycorticosterone concentration is markedly increased in peripheral blood [2, 3]. In a previous report [4], we examined the effect of several inhibitors of adrenal steroid synthesis on $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ enzymes purified from the bovine adrenal cortex mitochondria, and found that each inhibitor had a preferential site of action in steroid synthesis. Thus, information on the level of tissue cytochrome *P*-450 activities may assist in the choice and use of enzyme inhibitors in treating the functional adrenocortical tumors.

EXPERIMENTAL

Adrenocortical adenomas freshly excised from five patients diagnosed as having a primary aldosteronism on the basis of their clinical features and laboratory tests, and in whom the presence of aldosteronoma had been confirmed both surgically and histologically. Adrenal glands excised from patients with Grawitz's tumor uncomplicated by any other endocrine abnormality and with the normal plasma corticosteroid levels were used as the controls. None of the patients with primary aldosteronism had received inhibitors of adrenal steroid synthesis, and in those who had been treated with spironolactone the drug was discontinued for at least 2 weeks before surgery. None of the patients with the Grawtiz's tumor had been treated with anti-cancer drugs before surgery. The excised adenomata from patients with primary aldosteronism measured 1.3–1.8 cm in diameter.

Adrenal tissue (1 g) was combined with 2.4 ml of 30 mM potassium phosphate buffer (pH 7.4) and homogenized. Protein concentration was determined by the microbiuret method of Gornall *et al.* [6] with crystalline bovine serum albumin as the standard. The adrenal homogenate was then solubilized in potassium phosphate buffer, supplemented with 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Emulgen 913 and 1% cholate for 1 h at 4°C. *P*-450 content in the protein was measured by the Co-spectrum [7].

Assays

Steroid hydroxylase activities of individual *P*-450-linked enzymes in tissue homogenates were determined by a sensitive method recently developed in our laboratory [5]. The assay is based on the determination of the specific products of steroid monooxygenase reactions in the presence of sufficient amounts of the purified electron-donating components. Lipids and detergent were also supplemented so that the membrane associated *P*-450 is able to exhibit its maximum activity.

(1) Electron donor system. The electron transport system for the mitochondrial *P*-450 consisted of adrenodoxin reductase and adrenodoxin. Adrenodoxin and adrenodoxin reductase were obtained from bovine adrenal cortex and bovine adrenal cortex mitochondria as reported previously [8].

(2) Determination of the $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$ activities in adrenal homogenates. Tween 20 was added to [1α,2α(*n*)-^{3}H]cholesterol and the mixture incubated for 5 min to dissolve the cholesterol. After cooling the solution to room temperature, we placed it in an ice bath, and the adrenal homogenate (containing up to 0.2 mg of protein) was then added. The mixture was incubated at 37°C for 2 min, and the reaction was initiated by adding 15 mM NADPH. The reaction was carried out for 15 min at 37°C and terminated by the addition of 1.6 ml of methanol:chloroform (1:1). Pregnenolone (4-^{14}C) was added as the internal standard, and after the addition of 0.4 ml of distilled water and stirring

*Author to whom all correspondence should be addressed.

for 10 s, the mixture was centrifuged for 1 min. The organic solvent layer was removed by evaporation to dryness under a stream of nitrogen. The residue was dissolved in chloroform, spotted onto a silica-gel thin-layer chromatography plate, and chromatographed with authentic standards in a dichloromethane and ethyl acetate solvent system (6:4).

The P-$450_{11\beta}$ activity in the adrenal homogenate was assayed by the method described above, substituting [1α,2α(n)-^{3}H]DOC for [1α,2α(n)-^{3}H]cholesterol. Given the aforementioned instability of P-$450_{11\beta}$, 15 mM NADPH was added to the mixture before the addition of the electron donor compounds. The reaction was initiated with sufficient amount of homogenates (containing up to 0.2 mg of protein), and the mixture was incubated at 37°C for 3 min. The developing solvent was benzene:acetone (65:35). The position of each steroid was determined with a u.v. lamp, and the corticosterone formed was analyzed as reported previously [1]. In the present experiments, 15 mM ascorbic acid was added in order to exclude completely the effect of epinephrine from the blood or adrenal medulla. The effect of varying weight of adrenal homogeantes on purified bovine adrenal mitochondria obtained by the previous method [8] was also examined to test whether the homogenates contained sufficient levels of electron donors.

RESULTS AND DISCUSSION

The average activity of P-$450_{11\beta}$ in the control group was 9.0 nmol corticosterone/mg of protein/min, and in the adenoma group 55.6 ± 5.3 (68.2, 64.4, 54.2, 52.5 and 38.6) nmol corticosterone/mg of protein/min, indicating a significantly higher enzyme activity ($P < 0.01$) in the adenoma group than in the control (Fig. 1).

The average activity of P-450_{scc} in the control group was 6.2 ± 1.6 nmol pregnenolone/mg of protein/min, and in the 5 patients with adenomas 13.4 ± 2.0 nmol pregnenolone/mg of protein/min. The P-450_{scc} enzyme activity was slightly but significantly higher in the adenoma group than in the control group (Fig. 1).

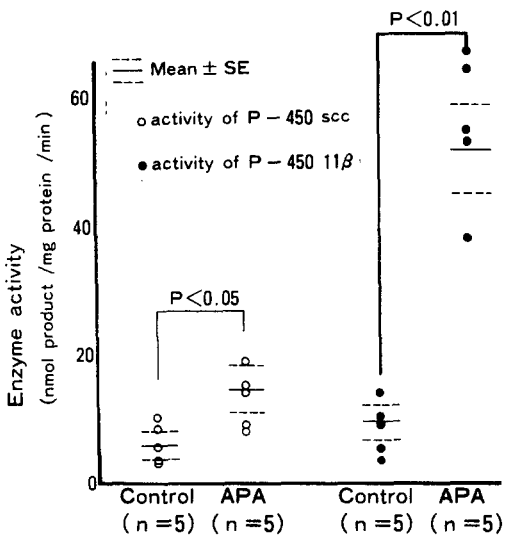

Fig. 1. P-450 activities of the adenoma tissues from 5 patients with aldosterone producing adenoma. Control adrenal tissues were obtained from the patients with the renal cell carcinoma who underwent unilateral adrenonephrectomy. P-$450_{11\beta}$ activity in aldosterone producing adenoma tissues was significantly higher than those in controls. P-450_{scc} activity was also significantly higher in aldosterone producing adenoma tissues but to a lesser degree as compared to P-$450_{11\beta}$.

There was no significant correlation between total enzyme activities (product of enzyme activities and wet weight of the tumor) and plasma aldosterone or cortisol concentrations.

The present study demonstrates that P-$450_{11\beta}$ enzyme activity in adenomatous tissues from patients with primary aldosteronism was approximately 6-fold higher than normal, as assessed by our reconstituted steroid monooxygenase system. We consider that enhanced mitochondiral P-450 activities may be a functional characteristic of aldosteronoma, although the P-450 activities of tissues from other adrenocortical tumors has to be investigated. The adrenal homogenates showed a similar increase in steroid production either in the presence or absence of the purified bovine adrenal P-450s, indicating that the homogenates contained sufficient amount of electron donors such as adrenodoxin and adrenodoxin reductase. Thus, the electron donor system is not a rate limiting step; on the other hand, we used sufficient purified electron-donating components in the homogenates to ensure maximum activities of the membrane associated P-450s [5].

The disturbance of aldosterone synthesis in aldosteronomata has not been clearly demonstrated as yet. In a previous report [9], aldosterone producing adenomas has been shown to contain cortisol and corticosterone at higher concentrations than those in control tissues, suggesting involvement of early steps of aldosterone synthesis. A disturbance of aldosterone synthesis at the corticosterone to aldosterone conversion step has also been suggested [10]. Although our results are in accordance with the previous report that the enzyme 11β-hydroxylase is also responsible for 18-hyroxylation in bovine adrenal [1], recently, Ogihara *et al.* [11] isolated aldosterone synthase P-450 from zona glomerulosa of rat adrenal cortex which is different from 11β-hydroxylase. Molecular cloning of cDNA revealed the existence of the two distinct genes in rat adrenocortical cells [12]. It may be interesting to speculate that 49K form [13] of P-450 or P-450aldo is abundantly produced in aldosteronomata. However, there is as yet no evidence to show the presence of two different forms of P-$450_{11\beta}$ in human adrenals.

The activity of mitochondrial P-450s in the altered aldosterone secretion following potassium intake, in sodium depleted state after angiotensin II stimulation have been demonstrated *in vivo*. The effect of these stimuli on the tissue P-450s in primary aldosteronism remains to be clarified.

Acknowledgements—This study was supported in part by a grant-in-aid from the Division of Intractable Diseases of the Ministry of Health and Welfare, Japan, 1989.

REFERENCES

1. Sato H., Ashida N., Suhara K., Itagaki E., Takemori S. and Katagiri M.: Properties of an adrenal cytochrome P-450 ($P450_{11\beta}$) for the hydroxylations of corticosteroids. *Archs Biochem. Biophys.* **190** (1978) 307–314.
2. Biglieri G. E. and Schambelan M.: The significance of elevated levels of plasma 18-hydroxycorticosterone in patients with primary aldosteronism. *J. Clin. Endocr. Metab.* **49** (1979) 87–91.
3. Kem D. C., Tang K., Hanson C. S., Brown R. D., Painton R., Weinberger M. H. and Hollifield J. W.: The prediction of anatomical morphology primary aldosteronism using serum 18-hydroxycorticosterone levels. *J. Clin. Endocr. Metab.* **60** (1985) 67–73.
4. Nagai K., Miyamori I., Takeda R., Sughara K. and Katagiri M.: Effect of ketoconazole, etomidate and other inhibitors of steroidogenesis on cytochrome P-450scc II catalyzed reactions. *J. Steroid Biochem.* **28(3)** (1987) 333–336.

5. Suhara K., Fujii K., Tani T. and Katagri M.: A sensitive method for quantitation of steroid hydroxylase activities of indivaisula P-450 in tissue homogenates. *J. Steroid Biochem.* **26** (1987) 113–119.
6. Gornall A. G., Bardawill C. J. and David M. M.: Determination of serum proteins by means of the biuret reactions. *J. Biol. Chem.* **177** (1949) 751–766.
7. Omura T. and Sato R.: The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239** (1964) 2370–2378.
8. Suhara K., Gomi, T., Sato H., Itagaki E., Takemori S. and Katagiri M.: Purification and immunological characterization of the adrenal cortex mitochondrial cytochrome P-450 proteins. *Archs Biochem. Biophys.* **190** (1978) 290–299.
9. Kaplan N. M.: The steroid content of adrenal adenomas and measurements of aldosterone production in patients with essential hypertension and primary aldosteronism. *J. Clin. Invest.* **46** (1967) 728–734.
10. Pham-Huu-Truang M. T., Duclos J. M., Pagny J. Y., Bogyo A., Leneuve P. and Girard F.: *In vitro* studies in primary aldosteronism: Baseline steroid production and aldosterone response to ACTH and angiotensin II. *Acta Endocr. (Copenh.)* **177** (1988) 135–144.
11. Ogihara T., Mitani F. and Ishimura Y.: Isolation of aldosterone synthase cytochrome P-450 from zona glomerulosa mitochondria of rat adrenal cortex. *J. Biol. Chem.* **5** (1989) 10635–10938.
12. Imai M., Shimada H., Okada Y., Matsushima-Hibiya Y., Ogishima T. and Ishimura Y.: Molecular cloning of a cDNA encoding aldosterone synthase cytochrome P-450 in rat adrenal cortex. *FEBS Lett.* **263** (1990) 299–302.
13. Lauber M., Sugano S., Ohnishi T., Okamoto M. and Müller J.: Aldosterone biosynthesis and cytochrome P-450$_{11\beta}$: evidence for two different forms of the enzyme in rats. *J. Steroid Biochem.* **26** (1987) 693–698.