Journal of Steroid Biochemistry, 1974, Vol. 5, pp. 185–190. Pergamon Press. Printed in Great Britain

IN VITRO PROGESTERONE METABOLISM IN LIVER FROM SPONTANEOUSLY HYPERTENSIVE RAT OR OPERATED RENAL HYPERTENSIVE RAT

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(*Received* 14 *June* 1973)

SUMMARY

Several causative factors are known to contribute to spontaneous hypertension in rats. The effect of high blood pressure on the activity of the hepatic enzymes metabolizing progesterone was studied *in vitro* in liver from spontaneously hypertensive rats or operated renal hypertensive rats. The *in vitro* capacity for the reduction of ring A in the livers of both types of hypertensive rats irrespective of sex, was much greater than that in the livers from normotensive control rats. Furthermore, the 4-ene-hydrogenase activity in the livers of pregnant rats was more than that in the livers of nonpregnant rats, and the rate of reduction of ring A of progesterone was greater in male rats after treatment with progesterone than in control male rats. These studies suggested that the increased capacity of hypertensive rat liver for *in vitro* reduction of progesterone might be due to the hypertensive state of the rats rather than being a cause of hypertension.

INTRODUCTION

Progesterone is known to be metabolized mainly in the liver in mammals, and its metabolism involves enzyme systems which reduce ring A of 4-ene-3-keto-steroids to the 5α or 5β stereoisomic forms. The many studies on the physiological importance of factors influencing 4-ene-hydrogenase activity have recently been reviewed by Schriefers[1]. Silah[2, 3] demonstrated that *in vitro* capacity for reduction of ring A of cortisol or corticosterone was greater in the livers of male rats with hypertension induced by renal operation than in that of livers from normal male rats. He suggested that hypertension, or some factor causing hypertension might in some way affect the enzymatic activity of the liver and possibly other tissues, including the adrenal, thus causing the alterations in the adrenocortical secretory pattern and in the metabolism of adrenocorticosteroids observed in hypertension. On the other hand De Nicola *et al.*[4] studied the *in vitro* metabolism of progesterone by the adrenals of spontaneously hypertensive rats and suggested that the adrenal cortex is not involved in the etiology of this form of hypertension. The present paper reports studies on the activities of hepatic enzymes specific for progesterone in the livers from spontaneously hypertensive rats.

MATERIALS AND METHODS

Chemicals

Reagent grade organic solvents were redistilled before use. [4-^{14}C]-Progesterone (specific radioactivity 29·3 mCi/mmol) obtained from the Radiochemical Centre, Amersham, and [1,2-^{3}H]-20α-hydroxy-4-pregnen-3-one (33·5 Ci/mmol) from New England Nuclear Corporation, Boston, Mass., were shown to be free from contamination by thin layer chromatography in toluene–ethyl acetate (1 : 1 v/v). Non-radioactive carrier steroids were purchased from Ikapharm Corporation, Israel. NADP was obtained from Sigma Chemical Co.

Animals

A colony of Wistar strain rats with spontaneous hypertension (SHR) were generously given by Dr. Okamoto, Kyoto University. Normal Wistar strain rats of similar wt. (180–250g) obtained from a local breeder served as normotensive control rats (NCR). The mean blood pressure of the SHR group was 194 $\pm$ 10 Torr. Renal hypertensive rats (RHR) were obtained by the procedure of Grollman[5]. Operations were performed under ether anaesthesia. Renal hypertension was induced by compression of the left

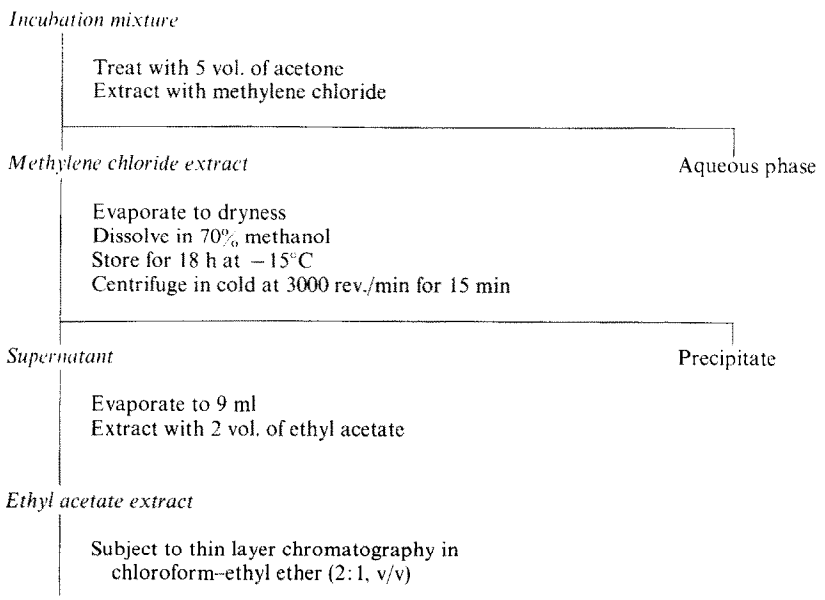

Fig. 1. Extraction and separation of steroids.

renal parenchyma with a cotton thread drawn taut over the pole and body of the kidney. Under ether anaesthesia about 10 days after the first operation the right kidney was removed. Control pregnant rats were obtained from the local breeder on the 11th day of pregnancy while SHR were mated in our laboratory. The day when vaginal sperm was found was designated day 0 of pregnancy. Rats were kept in a temperature-controlled room with 14 h of light and 10 h of darkness a day, and were given food and water *ad libitum*.

Enzyme preparation

Rats were killed by cervical dislocation. Livers were quickly removed, rinsed free of blood, chilled in ice-cold saline, dried on filter paper and weighed. Homogenates of liver were prepared in Krebs–Ringer phosphate buffer, pH 7·4 and centrifuged for 15 min at 800 ***g***.

Incubation

Incubation was carried out by the method of Wiest[6]. Enzyme preparation equivalent to 50 mg wet wt. of liver was incubated with 0·5 μCi of [4-^{14}C]-progesterone, 50 μg of progesterone, 1·2 μmol of NADP, 1·2 μmol of glucose-6-phosphate, 3 μmol of L-cysteine and 30 μmol of nicotinamide in a final volume of 3 ml of Krebs–Ringer phosphate buffer. The incubation was at 37°C with gentle shaking under air.

Extraction and separation of steroids

Steroids were obtained as described previously[7], following the procedure shown in Fig. 1. Progesterone and its metabolites were separated by thin layer chromatography on silica gel GF (Merck) using chloroform–ethyl-ether (2:1 v/v) as solvent system. Steroid products were located on the chromatogram using a radioactivity scanner. Unsaturated carrier steroids were detected on the chromatogram with U.V. light at 245 nm, and saturated steroids by the phosphomolybdic acid reaction.

Measurement of the radioactivity

The area on the chromatographic plates containing a separated steroid was marked out and scraped into scintillation vials. Radioactivity was determined by a liquid scintillation spectrometer. Radiochemical purity was assessed as described previously[8]. The labelled metabolites from experiments of each group were pooled, and rechromatographed on thin layer plates of silica gel. To the pooled eluates from thin layer plates, adequate amounts of carrier steroids were added, and crystals were allowed to form from the solvents as follows: carrier steroids added; progesterone, 5 mg, 5α-pregnane-3,20-dione, 5 mg, 5α-pregnan-3α-ol-20-one, 4 mg and 5α-pregnane-3α,20α-diol, 3·5 g, and solvent systems; progesterone, (1st) n-hexane, (2nd) heptane/benzene, (3rd) 70% methanol, 5α-pregnane-3,20-dione, (1st) acetone/hexane, (2nd) ethyl acetate, (3rd) methanol, 5α-pregnan-3α-ol-20-one, (1st) acetone/n-hexane, (2nd) heptane/benzene, (3rd) acetone/pentane, and 5α-pregnane-3α,20α-diol, (1st) ethanol/water, (2nd) methanol/water, (3rd) acetone/water. The quantities of 4-ene-3-ketosteroids were calculated from the optical

Table 1. Crystallization of steroid products*

Steroid product	Before crystallization	1st	2nd	3rd
Substrate: progesterone				
Progesterone	586000	545000	524000	537000
5α-Pregnane-3,20-dione	657000	659000	647000	663000
5α-Pregnan-3α-ol-20-one	518000	513000	525000	520000
Substrate: 20α-hydroxy-4-pregnen-3-one				
5α-Pregnane-3α,20α-diol	283000	241000	272500	240000

* Refer to text.
Values are expressed as d.p.m./mg steroid.

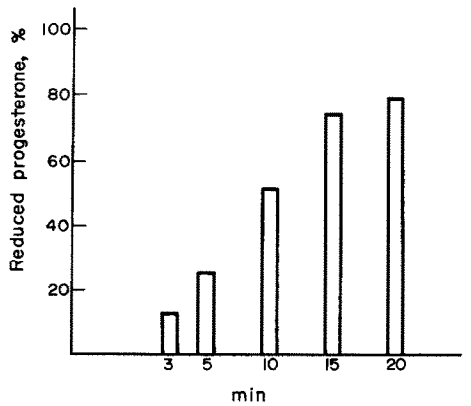

Fig. 2. Time course of progesterone reduction in the male NCR. Each flask contained 0·5 μCi of [4-^{14}C]-progesterone, 50 μg of progesterone, the supernatant fraction (800 *g*) of 50 mg of liver from normal male rats, 1·2 μmol of NADP, 1·2 μmol of glucose-6-phosphate, 3 μmol of L-cysteine and 30 μmol of nicotinamide in 3 ml of Krebs–Ringer phosphate buffer, pH 7·4.

densities of solution in ethanol at 220, 240 and 260 nm, using Allen's formula[9]. The concentration of 5α-pregnane-3,20-dione was calculated from the optical densities in concentrated sulfuric acid at 225, 245 and 265 nm, again using Allen's formula. The concentration of 5α-pregnan-3α-ol-20-one was calculated from the optical densities in concentrated sulfuric acid at 310, 330 and 350 nm, with Allen's correction. That of 5α-pregnane-3α,20α-diol was calculated from the optical densities in concentrated sulfuric acid 390, 420 and 450 nm, and the value of the optical density at 420 was corrected by Allen's formula. The metabolites isolated after each incubation were recrystallized to constant specific activity with authentic steroids (Table 1).

Recovery

The recoveries were 82–87% of the initial radioactivity. Data presented here are not corrected for losses incurred during the isolation of metabolites.

RESULTS

Rate of reduction of progesterone as a function of time

The time course of the reduction of progesterone in the male NCR is shown in Fig. 2. The rate of reduction of progesterone was linear for the first 15 min of incubation, and then decreased. Accordingly a 5 min incubation time was used.

Rate of reduction of progesterone as a function of enzyme concentration

Figure 3 shows the effect of the enzyme concentration (wet wt. of tissue) on the reaction in the male NCR. In the presence of excess substrate the reaction was proportional to the weight of tissue (25 mg to 100 mg) in the reaction mixture.

Reduction of progesterone by liver homogenates of NCR, SHR and RHR

The results are shown in Table 2. The reduction rate of progesterone by each two livers from female SHR or RHR were much higher than that of livers from NCR:

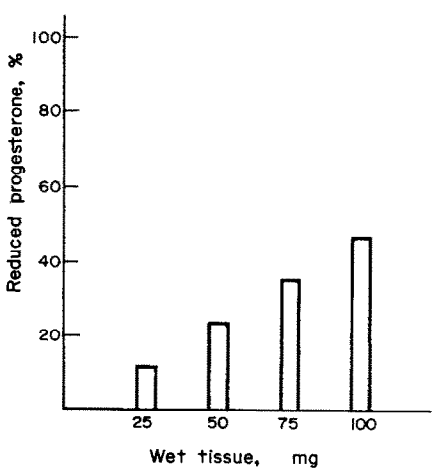

Fig. 3. Effect of enzyme concentration on progesterone reduction. Conditions were as for Fig. 2, except that its concentration was varied and mixtures were incubated for 5 min.

Table 2. Reduction of progesterone by liver homogenates of NCR, SHR and RHR

Groups*	Percentage of unchanged progesterone after incubation No. 1	No. 2
NCR (male)	76·6	75·4
NCR (female)	26·0	22·7
NCR (pregnant)	13·2	10·6
SHR (male)	57·9	59·6
SHR (female)	3·0	2·0
SHR (pregnant)	1·2	1·5
RHR (female)	1·3	1·6
NCR (male)†	57·1	55·7
NCR (male)‡	38·8	35·4

* NCR: normotensive control rat, SHR: spontaneously hypertensive rat, and RHR: operated renal hypertensive rat. Experiment: 2 rats for each group.

‡ Normotensive male rats treated with 5 mg of progesterone for 7 days.

† Normotensive male rats treated with 5 mg of progesterone for 7 days.

the percentages of unmetabolized progesterone remaining after incubation with preparation from each two female NCR, SHR and RHR were 24·3%, 2·5% and 1·4%, respectively (as mean values). On the other hand, the percentage of unchanged progesterone after incubation of livers from each two male NCR and SHR were 76·8% and 58·7%(as mean values). Thus the rate of reduction of progesterone in the liver was 3·1-fold higher in female NCR than in males and 2·3-fold higher in female SHR than in males. Further, 4-ene-hydrogenase activity by livers from NCR and SHR in pregnancy were determined. Rats in each group were killed on the 20th day of pregnancy. After incubation of preparations from each two NCR and SHR the amounts of unchanged progesterone were 11·9% and 1·3%, respectively (as mean values). Thus the rate of reduction of progesterone was much higher in the liver of pregnant rats.

To examine effects of progesterone on the 4-ene-hydrogenase activity in liver from male rats, normotensive male rats were injected with 1 mg or 5 mg of progesterone per day for 7 successive days and their livers were removed the day after the last injection. This treatment caused a significant increase in the reduction rate of progesterone by the liver (about 1·9 to 2·8 times the control value).

Products formed during progesterone metabolism by rat liver homogenates

Standard reaction mixtures were incubated for 5, 15, 30 and 60 min, and the percentage of progesterone converted to metabolites was measured. The results are shown in Fig. 4 and Fig. 5. Four main peaks were observed on thin layer chromatograms: I. progesterone, R_F 0·65; II. 5α-pregnane-3,20-dione, R_F 0·73; III. 5α-pregnan-3α-ol-20-one, R_F 0·56; and IV. 5α-pregnane-3α,20α-diol, R_F 0·13. As seen in Fig. 5, progesterone was metabolized *via* 5α-pregnane-3,20-dione, and then 5α-pregnan-3α-ol-20-one, to 5α-pregnane-3α,20α-diol. Under the present condition only a little 5α-pregnane-3α,20α-diol was found, so the experiment was repeated incubating samples with 20α-hydroxy-4-pregnen-3-one in place of progesterone. As seen in Fig. 6, a thin layer chromatogram of products obtained after incubation for five minutes with 20α-hydroxy-4-pregnen-3-one showed a high peak of 5α-pregnane-3α,20α-diol.

DISCUSSION

The identification of a colony of rats with spontaneous hypertension (SHR) was reported in 1962 and 1963 by Okamoto and Aoki spontaneous hypertension in these rats might be due to several factors, such as a neural factor, an endocrine factor or abnormality of vascular resistance.

Although the numbers of rats in each group examined are too small to come statistically to a conclusion, the present studies demonstrate that the *in vitro* 4-ene-hydrogenase activity for progesterone in the liver from SHR or RHR was greater than that in livers from NCR, and confirmed the results of Silah[2] on the livers of rats with hypertension induced by renal operation.

There have been several investigations on the low levels of urinary pregnanediol in toxemia of pregnancy characterized by hypertension, edema and proteinuria.

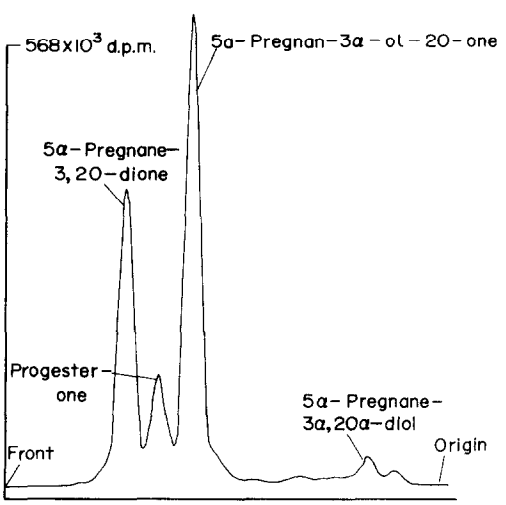

Fig. 4. Thin layer chromatogram of products of progesterone formed by rat liver homogenate (Radioscan). Solvent system: chloroform–ethyl-ether (2:1 v/v).

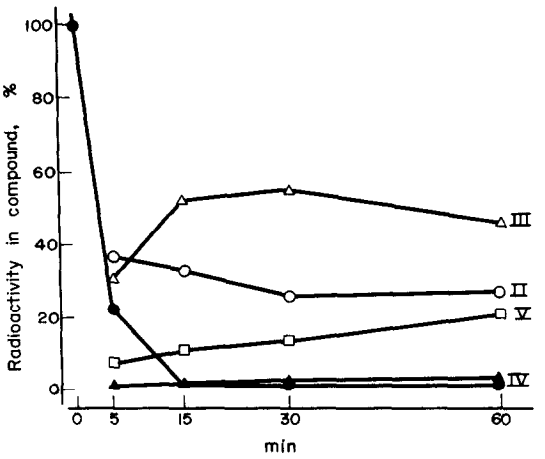

Fig. 5. Percentage of labeled progesterone converted to metabolites by homogenates of normal female rat liver as a function of the time of incubation. I: Progesterone; II: 5α-pregnane-3,20-dione; III: 5α-pregnan-3α-ol-20-one; IV: 5α-pregnane-3α20α-diol; and V: unknown metabolites. Conditions of incubation were as for Fig. 2.

It is generally thought that they are caused by reduced production and secretion of progesterone, its decreased conversion to pregnanediol due to an abnormality in its metabolism in the liver, or some inhibition of pregnanediol excretion in the urine. Using the isotope dilution method Vande Wiele *et al.*[12] observed that in these cases the rate of excretion of progesterone was 100 mg per day while in normal pregnant women it was between 265 and 500 mg. Tuchida[13] demonstrated that the concentration of progesterone in the placenta was less in toxemia of late pregnancy than in normal pregnancy and that synthesis of progesterone was very low. On the one hand, Armstrong[14] reported that progesterone lowered the blood pressure in rats and dogs with experimental hypertension, and in human with primary arterial hypertension, and that its effect in humans seemed to be due to naturiuresis. However, Mckay[15] thought that it was questionable whether the naturiuretic effect of the relatively enormous amount of progesterone secreted in normal pregnancy was actually responsible for the relatively low blood pressure observed in normal pregnancy and whether the diminished secretion of progesterone observed in toxemia actually resulted in sodium retention and so contributed to the elevated blood pressure in this syndrome. Silah demonstrated that 4-ene-hydrogenase activity in hypertensive rats was much greater than that in normal rats while rats that failed to develop hypertension had a normal enzyme level, and he presumed that the enzyme level could be related to the high blood pressure. Moreover, he concluded that the differences in the 4-ene-hydrogenase activity observed in hypertensive rats were due to differences in the content and/or activity of the enzyme in liver, because all his incubations were carried out in the presence of adequate quantities of NADPH. Yates *et al.*[16] found a close correlation between the 4-ene-hydrogenase activity of the liver and both the size of the adrenals and the magnitude of adrenocortical function, and concluded that there is a very close correlation between the size of the adrenal gland and the total hepatic capacity for *in vitro* hydrogenation of ring A. In the present experiments hepatic 4-ene-hydrogenase activity in female rats was demonstrated to be greater than that in male rats and it is well known that there is a sexual difference in the weight of the adrenals. As shown in Table 2, 4-ene-hydrogenase activity in livers of pregnant rats was more than in livers of non-pregnant rats. Moreover, the results in Table 2 indicated a remarkable increase in the rate of reduction of ring A on treatment of male rats with progesterone. When repeated doses of progesterone are given to women in the menopause the proportion of pregnanediol excreted increases to about 25% of the dose while after a single injection of progesterone into women after ovariectomy or during the follicular phase of the menstrual cycle, only about 10% is recovered in the urine as pregnanediol. Moreover, during pregnancy as much as 30 to 35% "additional" pregnanediol may be recovered following administration of progesterone[17]. These observations indicate that continued progesterone production or administration may modify its enzymatic reduction in the liver. Recently, Altman *et al.*[18] studied the induction of hepatic testosterone A-ring reductase by medroxy progesterone acetate (a derivative of progesterone) and concluded that the increase in activity is due to enzyme induction as a result

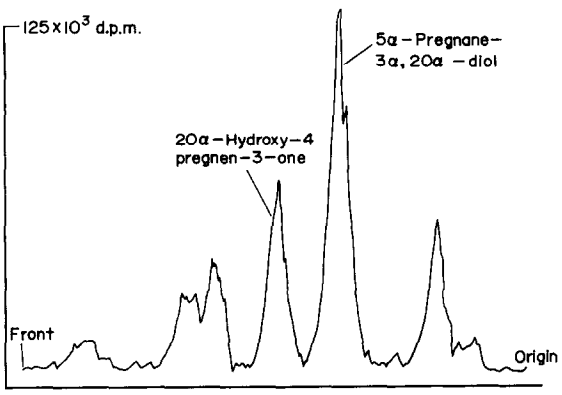

Fig. 6. Thin layer chromatogram of products of 20α-hydroxy-4-pregnen-3-one formed by rat liver homogenate (Radioscan). Conditions of incubation were as for Fig. 2, except that 0·2 μCi of 1,2-^{3}H-20α-hydroxy-4-pregnen-3-one and 50 μg of 20α-hydroxy-4-pregnen-20-one were added as substrate. Solvent system: toluene-ethyl acetate (1:1 v/v).

of enhanced synthesis of enzyme protein. In the present studies, including normotension and spontaneous and induced hypertension and in pregnant rats, the metabolism of progesterone was found to involve reduction of ring A. This occurred by 5α-reduction of the double bond at carbon 4, then 3α-reduction of the keto group at position 3, and finally reduction of the 20-keto group to give 5α-pregnane-3α,20α-diol. The last step was rate limiting. This pathway agrees with that indicated by Crane *et al.*[19].

Finally, the present finding that the capacity of hypertensive rat liver for *in vitro* reduction of progesterone increases may be due to the hypertensive state of the rat rather than being a cause of hypertension. This problem requires further investigation.

Acknowledgements—Spontaneously hypertensive rats were kindly supplied by Dr. K. Okamoto, Kyoto University.

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