J. steroid Biochem. Vol. 35, No. 1, pp. 121–125, 1990
Printed in Great Britain.

0022-4731/90 $3.00 + 0.00

CONCENTRATION DECREASE OF CORTICOSTEROID BINDING GLOBULIN (CBG) IN PLASMA OF THE MARE THROUGHOUT PREGNANCY

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(*Received* 25 *November* 1988; *received for publication* 22 *August* 1989)

Summary—A significant decrease of CBG binding capacity in plasma of the mare throughout pregnancy was demonstrated using equilibrium dialysis and gel equilibration methods. As indicated with immunoelectrophoresis experiments, the pregnancy related fall of CBG binding capacity was linked to an actual decrease in blood CBG concentration. This result contrasts sharply with data on most other mammalian species, with the exception of the gestating rhesus monkey.

INTRODUCTION

Transcortin or CBG, the major specific plasma protein which binds C_{21} steroids occurs in all vertebrate classes and in horses [1].

A significant rise in CBG concentration occurs during human pregnancy (to 2–3 times that in normal women) [2]. The administration of estrogens to nonpregnant women results in a similar rise [3] and it is now generally accepted that the increase in estrogen levels during pregnancy is responsible for the CBG rise.

In the pregnant mare plasma, estrogen concentrations remain at basal levels (0.2 nM) from the time of ovulation to Days 70–80 of gestation and then rise steadily with peak concentrations of unconjugated estrogens (1–2 nM) occurring around the seventh month of gestation and conjugated estrogens (1–2 μM) around the eighth month; thereafter levels of conjugated and unconjugated estrogens decline slowly to reach baseline values within a few hours after parturition [4].

We have previously reported [5] an absence of a specific plasma protein which binds C_{18} and C_{19} steroids (Sex Binding Protein or SBP) in horse plasma. Hence, unconjugated estrogens in peripheral mare plasma are in an unbound, biologically active form, and this raises the question as to their possible effect on CBG during pregnancy.

The work reported here was designed to determine the CBG binding capacity in the pregnant mare and to measure blood CBG content during gestation.

MATERIALS AND METHODS

Blood sampling

Blood samples were drawn from nonpregnant ($n = 7$) cycling thoroughbred mares in follicular or in luteal phase, and from pregnant ($n = 16$) thoroughbred mares at different stages of pregnancy. Blood was obtained by jugular venepuncture between 0900 and 1000 h and collected on EDTA. After centrifugation, the plasma was stored at −20°C until analysis.

Endogenous plasma steroids were removed by gently shaking the plasma for 1 h at room temperature after addition of Norit A charcoal (50 mg/ml). The plasma was obtained after two centrifugations at 2000 *g* for 10 min at 4°C.

Binding parameters

The unlabeled steroids were obtained from Roussel-Uclaf (Romainville, France); the [1,2,6,7-^{3}H]cortisol (SA, 100 Ci/mmol) from Amersham was shown to be homogeneous by thin-layer chromatography. Dialysis tubing (Arthur H. Thomas Co., Philadelphia, Penn., one-quarter inch) was obtained from Spectrum Medical Industries (Newark, Del.) and Sephadex G-25 fine from Pharmacia (Uppsala, Sweden). Binding experiments were performed by an equilibrium dialysis method and by batchwise equilibrium dialysis technique [5]. For dialysis experiments 1 ml diluted plasma (1:50) was placed inside the bag, and the [^{3}H]cortisol was placed outside in 3.5 ml Tris buffer (0.01 M Tris–HCl buffer, pH 7.4). In competitive experiments, the unlabeled and labeled steroids were both placed outside the bag. To each dialyzing system were added various amounts of nonradioactive steroid, ranging from 10^{-10} to 10^{-7} M, and a fixed amount of labeled steroid (1×10^{-10} M). All binding experiments were performed at 4°C with stirring for 48 h.

The batchwise gel equilibration technique [6] was also used for the determination of binding constants. 200 mg of Sephadex G-25 fine was allowed to settle overnight in 1 ml 0.15 M phosphate buffer, pH 7.4, at room temperature. 130 pg of labeled cortisol in phos-

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phate buffer were added to each test tube. We then added 0.5 ml plasma (diluted 1:10) and increasing quantities of unlabeled cortisol (up to 80 ng), total phosphate buffer volume being adjusted to 2 ml. One set of control tubes was used for the determination of the partition coefficient of cortisol between the external and internal phases of the Sephadex suspension. Equilibration was achieved by shaking all tubes horizontally at 37°C for 1 h. After allowing the Sephadex to settle for 30 min, the radioactivity of 0.5 ml supernatant was determined.

Experimental data were computed using a computer program [7] and by graphic analysis [8]. The statistical significance of differences between mean values of binding constants was tested using Student's t-test.

Relative quantification of CBG

Laurell's two dimensional immunoelectrophoresis using an antirabbit whole horse serum (Z 309 DAKOPATTS) was performed [9]. Plasma samples were incubated overnight with [^{3}H]cortisol (10^{-7} M) prior to being subjected to immunoelectrophoresis. At the end of the migration, the plates were dried and exposed to an X-ray film (^{3}H-Ultrofilm LKB, Stockholm, Sweden) for 7 days, and then stained with Coomassie blue or Sudan Black.

RESULTS

Mare plasma bound cortisol with high affinity ($K_A = 2 \times 10^9\ M^{-1}$) and limited capacity (Fig. 1). The specificity of mare CBG was assayed by examining the cross-reactivity of different steroids at 50 percent displacement of [^{3}H]cortisol. Except for corticosterone (10% displacement) and prednisolone (5%), the cross-reactivity in respect of the other steroids

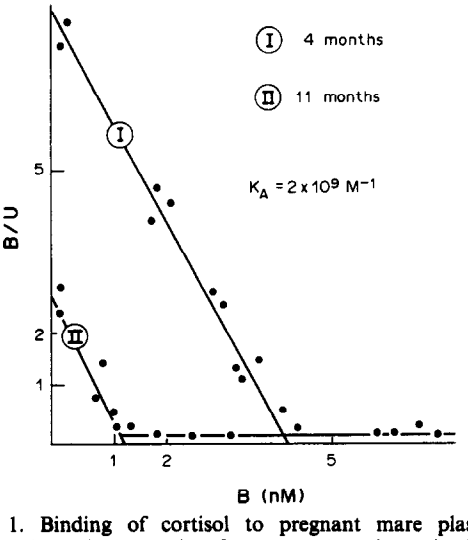

Fig. 1. Binding of cortisol to pregnant mare plasma (4 months and 11 months of pregnancy) as determined by equilibrium dialysis experiments. Scatchard-type plots of total binding; B/U: concentration of bound steroid/concentration of unbound steroid; plasma diluted 50-fold.

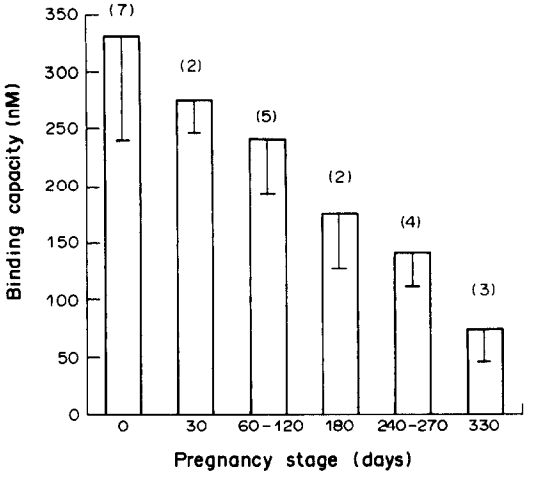

Fig. 2. Binding capacity of cortisol (nM) in nonpregnant and pregnant mares; mean ± SD; numbers in parentheses: number of animals. Each determination was made on a sample obtained from one animal.

tested (progesterone, testosterone, dexamethasone, estradiol-17β) was less than 1% at 50% displacement (data not shown).

Figures 1 and 2 show a significant decrease of CBG binding capacity during pregnancy. Mean binding capacities were significantly different ($P < 0.01$) between: (1) nonpregnant mares and pregnant mares at 240–270 days or 330 days of pregnancy and (2) pregnant mares at the beginning of pregnancy (30 days) and those at the end of pregnancy (9 or 11 months). They were not significantly different ($P > 0.05$) between nonpregnant mares and pregnant mares up to the 180th day of pregnancy or between pregnant females at the beginning of pregnancy and at 180 days of pregnancy.

For the all blood samples studied (from pregnant and nonpregnant females) the differences in K_A values were not statistically significant ($P > 0.05$).

The changes in the plasma CBG binding capacity during pregnancy are also illustrated by agarose gel electrophoresis (Fig. 3).

To compare the amount of CBG in blood samples from mares at 1 month and 9 months of pregnancy, two dimensional immunoelectrophoresis was performed. The second-dimensional gel using rabbit anti-whole horse serum allowed us, after autoradiography, to identify the CBG precipitation arc. In Fig. 4, the lower labeled arc representing plasma levels at the 9th gestational month illustrates the decrease in CBG occurring during pregnancy.

DISCUSSION

The CBG binding capacity in nonpregnant mare (0.33 μM) was found to be lower to values reported in woman (0.7 μM), mouse (0.9 μM) or guinea-pig (2–3 μM), higher to values in badger (0.07 μM) and sow (0.05 μM); and similar to values in cow (0.28 μM) [1, 10–12]. The horse CBG binding ca-

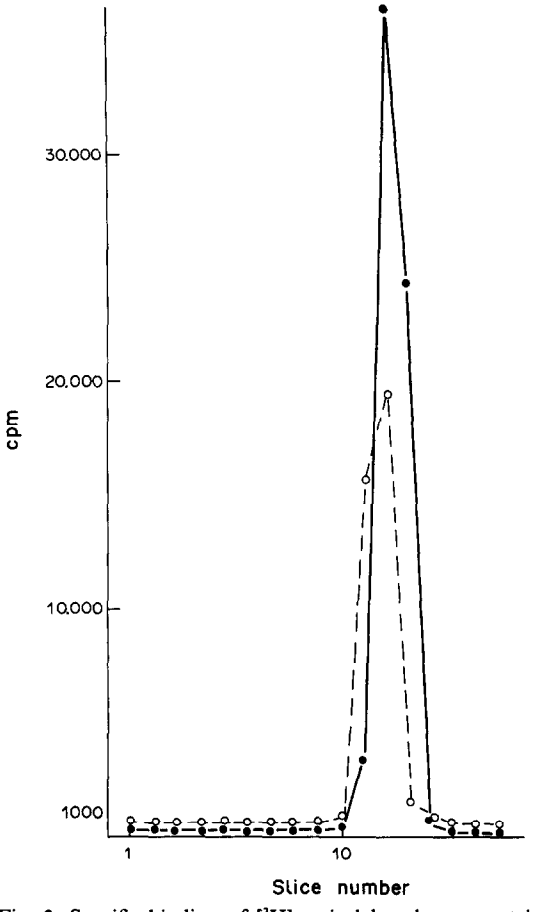

Fig. 3. Specific binding of [^{3}H]cortisol by plasma protein from mares at 1 month (●——●) and 9 months (○---○) of pregnancy. Blood samples were first incubated with 2×10^{-7} M [^{3}H]cortisol, and 5 μl was analyzed by electrophoresis in 1% agarose gel in barbital buffer, pH 8.6, at 10 V/cm for 3 h. (Anode to the right). Gels were then sliced into segments and placed into counting vials.

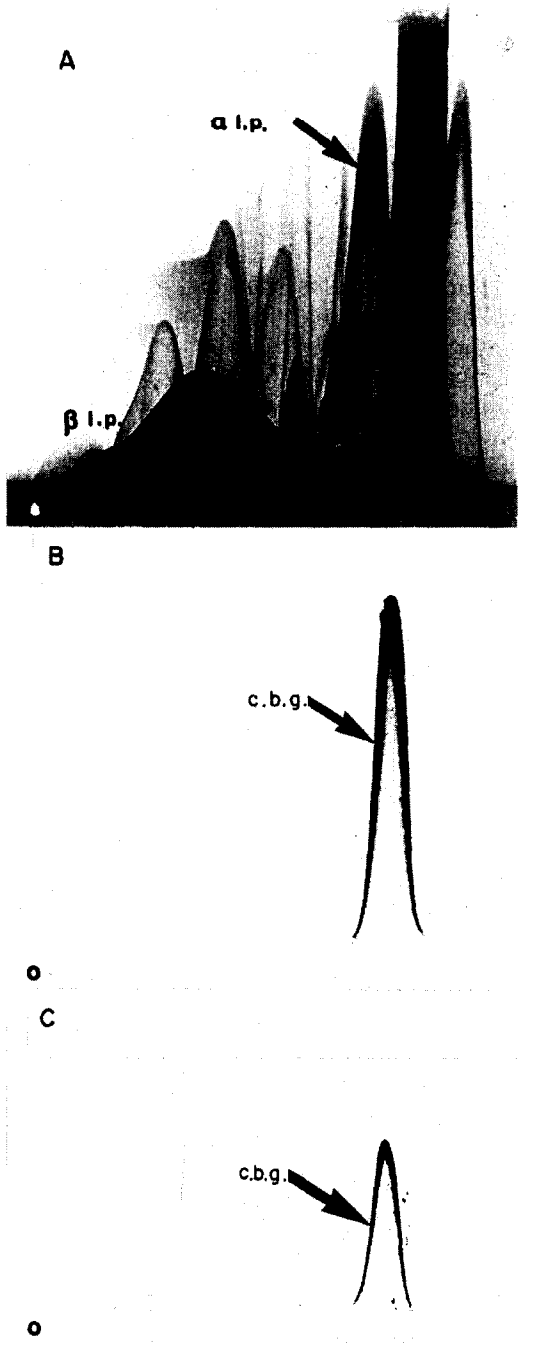

Fig. 4. Two dimensional electrophoresis of mare plasma at 1 month (B) and 9 months (C) of pregnancy. Blood samples were first incubated with [^{3}H]cortisol (2×10^{-7} M) and 5 μl was analyzed. The first dimensional run was done at 10 V/cm for 3 h, anode to the right. The immunoelectrophoretic run was done at 2 V/cm for 16 h in agarose gel against 10% (v/v) antirabbit whole horse serum followed by autoradiography (7 days of exposure). After the run 2-D, gels were stained with Coomassie Blue or Sudan Black (A); l.p. arcs (α and β) were positive for lipid.

pacity determined in our study was found slightly higher than the value (0.23 μM) obtained by Irvine and Alexander[13] by the use of the charcoal adsorption technique. Horse CBG specifically binds cortisol. Murphy[14] had previously utilised this property to develop a specific and sensitive radioligand assay for the measurement of human plasma cortisol. In specificity the radioligand assay appears superior to the radioimmunoassay that utilizes antisera to cortisol 3-BSA [15].

Our data indicate a significant pregnancy-related decrease of transcortin in mare plasma. The drop in CBG binding activity is statistically demonstrated for the last period of pregnancy. The immunoelectrophoresis results show that the fall of CBG binding capacity was linked to an actual decrease in blood CBG concentration. The pregnancy-related response of plasma CBG in the mare contrasts sharply with data on most other mammalian species with the exception of the gestating *Pteropus giganteus*, *Nycticebus coucang* [16], and the rhesus monkey which has been shown to exhibit a 30% decrease of CBG binding capacity [17]. On the other hand, women, rabbits, guinea-pigs, mice, badgers and sows, all show considerably elevated CBG binding capacities during gestation [1, 16, 18–21] and in other species such as the rat and cow, CBG levels remain

unaltered during the same period [11, 22]. While in pregnant women, the increase in CBG is generally linked to the rise in blood estradiol [3, 10], it is important to note that in the pregnant mare, the CBG levels began to fall before the rising estrogen levels had peaked. Moreover, after the drop in estrogen levels, the plasma estrogen content still remained higher than in the nonpregnant mares. Since the CBG concentration began to decrease when the plasmatic estrogen concentration enhanced, and went on to decrease after the drop of estrogens, the elevated and decreased estrogen levels could be considered as unresponsible for the CBG decrease.

Contrary to humans whose total cortisol concentration rises above that of nonpregnant women after the 11th week of gestation [23] plasma corticosteroid concentrations in the pregnant mare show no change [24]. Dunn *et al.*[25] report that the increased CBG levels in pregnant women (1.7 vs 0.7 μM) cause, in fact, a 50% decrease in the percent unbound cortisol compared to normal women. In the mare, the pregnancy-related decrease in plasma CBG must reinforce the action of cortisol which is carried by the blood stream in a higher free state.

Until recently, it has generally been thought that the only role of CBG is to modulate the concentration of plasma free C_{21} steroids that can diffuse passively from the blood into target cells. Since steroid uptake by target cells results in decreased concentrations of plasma free hormones, the steroid dissociates from the CBG bound fraction in order to maintain its availability. The binding of the C_{21} steroid by CBG renders it biologically inactive. However, some few recent studies have suggested that a distinct mechanism must exist for enriching intracellular steroid concentrations; data on the presence of a specific binder for CBG on the membrane of target cells, and on the intracellular location of CBG in glucocorticoid target cells indicate that CBG may play a more active role than previously believed [26–30].

The species-specific variations in CBG levels observed during pregnancy could reflect not only changes in the rate of synthesis of CBG, but also alterations in the CBG uptake by the target cells. A possible explanation of the decrease in CBG levels observed in the pregnant mare may be an increase in cellular binding sites and thus an increase in CBG uptake. Only future experiments will reveal whether the lower levels of CBG in the pregnant mare signify an enhanced or a decreased glucocorticoid action.

Acknowledgement—Mr P. Ranguis is thanked for the illustrations.

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