Cortisol Binding to Proteins in Plasma in the Human Neonate and Infant

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Extract

The binding of cortisol to plasma proteins was studied by a methodology allowing the determination of the apparent affinity constant (K_a) and the specific binding capacity (N_s) for transcortin in different blood compartments in normal pregnant women at term, in the umbilical cord blood, and in normal neonates and infants throughout the first year of life. Removal of endogenous steroids appeared to be necessary for these binding studies, especially in the cord plasma. Both an equilibrium dialysis and an adsorbent technique were used, inasmuch as it has been established that they yield similar results for corticosteroid-binding globulin (CBG) binding parameters.

The high affinity binding of cortisol, attributed to transcortin, was found to have the highest capacity in plasma from pregnant subjects ($N_s = 1.16 \pm 0.19 \times 10^{-6}$ M), whereas the fetal compartment (umbilical cord) exhibited low values ($0.25 \pm 0.03 \times 10^{-6}$ M). Thus a physiologic barrier to the transplacental passage of transcortin resulted in a large concentration gradient in cortisol-specific binding sites between the fetal and maternal compartments. No significant differences were found for transcortin capacity between venous uterine plasma and peripheral plasma in three subjects studied during cesarean section. A possible physicochemical difference between maternal and fetal transcortin appeared unlikely, because in all blood compartments studied, the apparent affinity constant, K_a , was similar ($5-8 \times 10^8$ M⁻¹ at 4°), and the behavior of the specific cortisol binder in polyacrylamide gel electrophoresis was the same in all cases.

After birth, although the transcortin K_a for cortisol was still in the same range, N_s remained low during the first month of life ($0.23 \pm 0.07 \times 10^{-6}$ M); after which an increase toward normal adult values was seen, with a rather large variation between individuals from 2-12 months of age (mean $0.48 \pm 0.10 \times 10^{-6}$ M). Children above 1 year exhibited adult values for N_s ($0.51 \pm 0.03 \times 10^{-6}$ M).

Speculation

From the similarity of values found for transcortin K_a and electrophoretic behavior, the physicochemical properties of the glycoprotein cortisol binder are likely to be the same both in the maternal and fetal compartments, although final evidence of chemical identity is not available. Thus, the different binding capacity between these compartments might be attributed to a different concentration in circulating transcortin. This concentration gradient may play a role in the transplacental transfer of corticosteroids during pregnancy and would favor passage toward the maternal compartment. The low transcortin level in the neonatal period might be

attributed to a low biosynthesis capacity of the liver in the neonate, although nothing is known about the clearance rate of transcortin in the neonatal period. The transcortin level increases toward adult values during the first year of life and this would suggest either a maturation of the liver biosynthesis enzymatic systems or a biosynthesis stimulation. A possible contribution of a pituitary factor might be suggested on the basis of experimental work in the rat, but the meaning of this increase in cortisol binding capacity in plasma remains to be elucidated.

Limited capacity (specific) high affinity binding of cortisol to transcortin (CBG) in human plasma and other mammalian species is well established; human adult CBG has been isolated by several groups and its physicochemical as well as binding properties have been well described (36). Since then, cortisol binding to plasma proteins has been investigated in normal and various pathologic conditions in the human adult (36). Low values of binding capacity have been reported in human cord plasma (14, 32). However, in most of the studies in the human, only the estimation of the binding capacity has been considered. On the other hand, it has been shown that the newborn rat also has a low plasma cortisol binding capacity, rising to the male or female adult values within the first 4-6 weeks of life (36) and that the rhesus monkey shows a progressive increase of plasma cortisol binding capacity after birth until the time of sexual maturation (5).

This work is concerned with the study of the plasma cortisol binding parameters in the human newborn period (K_a and CBG N_s), both by equilibrium dialysis and by a more rapid adsorbent technique. The possibility of physicochemical differences between neonatal and adult CBG was examined (K_a , polyacrylamide gel electrophoresis). In addition, CBG binding parameters were studied in a number of individual neonates and infants during the first year of life.

EXPERIMENTAL PROCEDURE

PLASMA SAMPLES

Fourteen normal adults who were not taking any therapy were studied: 4 adult males and 10 pregnant females at term. Venous blood, 10-20 ml, was withdrawn into heparin and immediately centrifuged. The resulting plasma was studied on the same day or kept frozen at -20° before processing. In three additional cases, it was possible to obtain blood samples during cesarean section (carried out for obstetrical reasons and resulting in birth of normal infants). In these cases, cortisol binding could be studied in antecubital-peripheral plasma, in

plasma from the uterine vein obtained by puncture during surgery, before uterostomy, and also from the umbilical cord after its section. Cord plasma was otherwise obtained by collection after vaginal delivery and after section of the umbilical cord; this was a mixture of arterial and venous fetal plasma.

Twenty normal neonates and infants (12 males and 8 females) were studied; all were born at term, after a normal pregnancy terminated by vaginal delivery.

REAGENTS

Steroids were obtained from Mann Research Laboratory (38); 1,2-[³H]cortisol (specific activity 35 Ci/mmol) was from New England Nuclear and purified before use by thin layer chromatography (CHCl₃-methanol, 8:2). Visking tubing (18/32) was used for dialysis after extensive washing as described by Westphal (35). Activated charcoal (Norit A) was from Sigma; all other reagents, A grade, were purchased from Prolabo or Merck.

Removal of Endogenous Steroids from Plasma. This was carried out according to the method of Heyns *et al.* (22) by continuous shaking with activated charcoal (50 mg/ml plasma) for 2 hr at room temperature, followed by centrifugation and filtration of the supernatant.

Determination of Plasma Corticosteroids. The assay was carried out by competitive protein binding according to the method of Murphy (28) using dog plasma as the source of binding protein.

Equilibrium Dialysis. This was carried out at 4° for 48 hr in phosphate buffer, pH 7.4, 0.155 M, according to the method of Westphal (35). The dialysis bags usually received 2 ml 1:62.5 diluted plasma. In 10 ml outside buffer, 1:25 μ Ci tritiated cortisol and various amounts of cold steroid were dissolved so to achieve 15 different final concentrations of total steroid ranging from 10⁻¹⁰ to 10⁻⁷ M.

Adsorbent Technique. This technique was also used for the separation of the unbound and bound forms of the steroid after development of the binding equilibrium at 4°. The technique described by Pegg and Keane (29) was followed, the only modification being that instead of Dextran-charcoal, the adsorbent suspension used was made of 1 volume of 1% Norit A + 1 volume of 0.05% polyvinylpyrrolidone, both in phosphate buffer, pH 7.4, 0.155 M. Each series included duplicate tubes to which buffer was added instead of the charcoal suspension for the counting of the total radioactivity (TR) and three tubes without addition of proteins, for the determination of the nonadsorbed radioactivity (NAR). For each concentration of cortisol, the bound radioactivity (B_R) in the supernatant was measured and the mass of cortisol bound (B_M) calculated according to

$$B_M = \frac{B_R - NAR}{TR} \times (M + m + e)$$

where M = cold cortisol added; m = mass of the added tritiated cortisol; e = mass of the endogenous competing steroid (e was usually neglected since removal of the endogenous steroids was carried out previously).

Polyacrylamide Gel Electrophoresis. Electrophoresis was done in 5 mm (inner diameter), 8 cm long Pyrex tubes according to the method of Davis (13). The separation gels were 7.5% in acrylamide and 0.2% in bisacrylamide concentrations; stacking gels were 2.5% in acrylamide and 0.2% in bisacrylamide concentrations. Bromphenol blue was added to each sample as a tracking dye and to allow the measurement of the R_F values for the proteins of interest (12). Staining of control separations was carried out by amido black 10B.

Labeling of the plasma binding proteins by their radioactive ligand was carried out as follows: the radioactive steroid (0.1 μ Ci) was evaporated to dryness and 100 μ l plasma were added.

After 30 min at 37°, the mixture was incubated for 1 hr at 0°; 100 μ l 60% sucrose solution in the upper electrophoresis buffer, containing bromphenol blue (1%), were added. Fifty microliters were used immediately for the electrophoresis. After completion of the run, the gels were rapidly cut into 2-mm thick slices.

Liquid Scintillation Counting. Counting was carried out with an Intertechnique SL-30 counter, using the external standardization method. The scintillation mixture was toluene-Triton X-100 (2:1), Permablend II, 5.5 g/liter. This mixture insured an overnight dissolution of the polyacrylamide gel slices.

Results of Binding Experiments. Results were plotted according to the procedure of Scatchard (33) for the determination of K_a and N_s. Some of the results were represented as Lineweaver-Burk (25) and Baulieu-Raynaud (4) plots, using a manual approach.

RESULTS

REMOVAL OF PLASMA ENDOGENOUS STEROIDS

When an untreated umbilical cord plasma was studied, no high affinity binding was obvious on a displacement curve, the radioactive steroid binding being impaired by the presence of large amounts of endogenous steroids and/or possibly other interfering substances (Fig. 1). It was thus necessary to remove this bulk of interfering compounds; the charcoal treatment changed the results drastically and thus rendered possible the study of the present binding systems (Fig. 1). Electrophoresis of plasma before and after charcoal treatment did not show

Fig. 1. Cortisol binding and displacement curve under the same conditions (plasma final dilution 1:62.5) for the same plasma before and after charcoal treatment and removal of endogenous binding inhibitors.

any gross modification of the qualitative and quantitative patterns of protein in plasma. At the same time, however, the concentration of the "total corticosteroids," measured as cortisol by competitive protein binding, dropped drastically from 19 μg to 3.1 $\mu\text{g}/100$ ml plasma in the example in Figure 1. Heyns *et al.* (22) reported similar residual values after charcoal treatment of 30 plasma samples (2.6 ± 1 $\mu\text{g}/100$ ml plasma).

EQUILIBRIUM DIALYSIS VERSUS ADSORBENT METHOD

The results obtained by the two methods with six different plasmas are reported in Table 1. The difference between paired results by the two methods was in all cases less than 10%. Despite the fact that the adsorbent technique is not thermodynamically valid since it is a nonequilibrium method, the values obtained for K_a and N_s for CBG were similar with both techniques under the conditions used in this work. As far as the reproducibility of this adsorbent technique is concerned, our results yielded figures similar to the values reported by Pegg and Keane (29) with a variation coefficient of less than 10%.

CBG LEVELS IN FETAL AND MATERNAL COMPARTMENTS

In three cases, cortisol binding could be studied by equilibrium dialysis at 4° in cord plasma and in the uterine vein and peripheral plasma from the mother (Table 2). Similar K_a values were found in all cases ($\sim 10^8$ M^{-1}), which indicated that a high affinity binder was present in both compartments, with the K_a expected for the association of cortisol-CBG. N_s was similar in all of the plasmas of maternal origin, and showed no difference between the peripheral level of CBG and the level in the uterine vein. By contrast, the capacity of the specific binder in cord plasma was four- to fivefold less than in the maternal compartment.

After labeling of the plasma proteins by [^3H]cortisol, polyacrylamide gel electrophoresis showed, both for maternal and fetal plasma, the presence of the same cortisol-binding material as a well defined radioactive peak with an R_F identical in both cases (0.58) (Fig. 2). Furthermore, in both cases, this radioactive peak was diminished and then completely erased by addition of increasing amounts of nonradioactive cortisol, corticosterone, or progesterone.

CORTISOL BINDING IN NEONATE AND INFANT

The adsorbent method and the calculation using the Scatchard plot were carried out for the determination of the

Table 1. Comparison of results obtained by equilibrium dialysis (ED) and adsorbent technique (AT) for corticosteroid-binding globulin binding capacity (N_s) and apparent association constant (K_a), at 4°, in different human plasma

Plasma source	Technique	N_s , μM	Difference, %	K_a , 10^8 M^{-1}	Difference, %
Normal neonate (8 days)	ED	0.26	4.6	8.7	1.1
	AT	0.25		8.6	
Normal infant (4 mo)	ED	0.40	3.1	7.2	8.9
	AT	0.41		6.5	
Normal adult male	ED	0.46	4.6	5.0	3.2
	AT	0.48		4.8	
Normal pregnancy (term)	ED	1.38	9.6	5.0	10.0
	AT	1.25		4.5	
Normal pregnancy (term)	ED	1.22	8.1	13.3	8.4
	AT	1.33		14.5	
Normal pregnancy (term)	ED	1.10	2.2	7.9	10.2
	AT	1.12		8.4	
Mean $\pm$ SD		5.3 ± 2.6		6.9 ± 3.5	

Table 2. Corticosteroid-binding globulin binding parameters for cortisol, in different fetal and maternal compartments (equilibrium dialysis, 4°)¹

Plasma source	N_{s1} ²	K_{a1} ³	N_{s2}	K_{a2}	N_{s3}	K_{a3}	N_s (mean $\pm$ 1 SD)	K_a (mean $\pm$ 1 SD)
Maternal peripheral vein	1.13	6.9	1.25	4.9	1.33	6.0	1.24 $\pm$ 0.09	5.93 $\pm$ 0.82
Uterine vein	1.22	5.2	1.07	9.9	1.29	5.5	1.20 $\pm$ 0.09	6.86 $\pm$ 2.15
Umbilical cord	0.29	4.3	0.22	5.3	0.25	5.7	0.25 $\pm$ 0.03	5.10 $\pm$ 0.59

¹ Numeral with N_s and K_a indicates three different pregnant subjects.

² Binding capacity, micromoles of cortisol per liter of plasma.

³ Apparent association constant, 10^8 M^{-1} .

Fig. 2. Polyacrylamide gel electrophoresis of maternal and cord plasma after labeling with tritiated cortisol. Plotting of the radioactivity along the gel after the electrophoretic separation showed in both cases a major peak of the same mobility (R_F 0.58) attributed to plasma corticosteroid-binding globulin (CBG). The small peak (R_F 0.69) had the electrophoretic mobility of human serum albumin. The CBG peak was suppressed by addition of 10^{-6} M of unlabeled cortisol. R_F values were measured by reference to the band given by the bromphenol blue, which was used as the tracking dye.

CBG binding parameters (K_a , N_s) in plasma from different normal neonates and infants. To compare our results with values reported previously by other workers using various experimental methods, four normal adult males were studied.

The average values obtained were 5.7×10^8 M^{-1} for K_a and $0.50 \pm 0.03 \times 10^{-6}$ M for N_s (Table 3):

Ten normal pregnant women at term gave average values ($1.16 \pm 0.19 \times 10^{-6}$ M for N_s and $8.2 \pm 3 \times 10^8$ M^{-1} for K_a), also in agreement with previously established data.

By contrast, the values found for the umbilical cord plasma studied were similar to the adult values with regard to the

apparent affinity constant ($5.1 \pm 0.6 \times 10^8 \text{ M}^{-1}$), but the binding capacity was half that of the normal male adult ($0.25 \pm 0.03 \times 10^{-6} \text{ M}$), confirming the previous findings by De Moor (14) and Sandberg (32).

The binding capacity remained low during the first month of life, the average value being $0.23 (\pm 0.07) \mu\text{M}$. A rise of this value toward adult level was seen in the following months of life (Table 3), and, after 1 year of age, all of the children studied exhibited adult values for N_s as well as for K_a . Thus, the neonatal low plasma CBG concentration appeared to increase slowly toward the adult level.

However, the variations within individuals appeared rather large in this process and the average value obtained from 2–12 months of age was similar to the adult figure (0.48 versus $0.50 \mu\text{M}$), the standard deviation being much larger (0.10 versus 0.03). However, all values above 2 years of age are in the adult range (Table 3).

DISCUSSION

Equilibrium dialysis is the thermodynamically valid method of choice for steroid binding study (see Reference 36 for references). Several problems are encountered in the use of adsorbents and have recently been thoroughly investigated in the case of dextran-coated charcoal suspension (9). Pegg and Keane (29) have already introduced a dextran-charcoal technique for the determination of the CBG binding parameters in human adult plasma with satisfactory results. Our data obtained on the same samples by parallel equilibrium dialysis and the adsorbent technique showed that indeed the adsorbent method, although not valid for thermodynamic studies, was useful for comparative studies of binding properties in well defined biologic mixtures.

A Scatchard plot was used in all cases for the CBG K_a and N_s evaluations (Fig. 3). Typically, two straight lines could be quite easily defined and the steepest one was used for the estimation of the CBG binding parameters. Several procedures have been proposed for a more accurate graphic evaluation of the specific system when it is present together with a nonspecific, high capacity one (19, 30). However, in our case, two binding systems were well defined and a graphic construction according to the method of Rosenthal (30) brought only negligible differences in the results for K_a . In a few cases, other types of graphic representations were used. The reciprocal plot (25) and the proportional graph (4) yielded very similar results from the same experimental data (Fig. 3).

The "specific" cortisol binding moiety present in the neonatal plasma had a similar affinity constant and the same mobility on polyacrylamide gel electrophoresis as the adult CBG and, to our knowledge, no difference in physicochemical properties between adult and neonatal CBG has been reported. On the other hand, the presence of competitive inhibitors in the neonatal plasma which were not removed by the charcoal treatment would alter CBG K_a but not N_s values. Other types of inhibitors might explain this low N_s with normal K_a , but the more likely explanation is that of a low circulating CBG level. By contrast to the elevated level in the maternal compartment, this low level on the fetal side raises several problems, as follows.

CORTISOL TRANSPLACENTAL TRANSFER NEAR TERM

Cortisol exchange between the maternal and fetal compartments has been demonstrated in both directions (6, 26) with an apparent predominance from fetus to mother (2, 3, 27). The existence of a placental diffusion barrier has been documented in animal experiments (7, 15). Our studies were carried out at birth and our results may reflect the fetal situation near term. There are some major differences between the fetal and maternal vascular compartments, as far as specific cortisol binding is concerned. On the maternal side, the CBG

Table 3. *Binding capacity*

Subjects		$N_s (10^{-6} \text{ M})$	$K_a (10^8 \text{ M}^{-1})$
Normal adult males			
1		0.48	4.9
2		0.51	5.9
3		0.56	7.5
4		0.46	4.6
Mean ± 1 SD		0.50 ± 0.03	5.7 ± 1.1
Normal pregnant females (term)			
1		0.99	7.9
2		1.25	4.5
3		1.45	3.2
4		0.89	6.5
5		1.05	10.2
6		1.33	14.5
7		0.98	9.3
8		1.47	10.2
9		1.13	8.4
10		1.03	7.6
Mean ± 1 SD		1.16 ± 0.19	8.2 ± 3.0
Umbilical cords			
1	M	0.25	5.7
2	F	0.29	4.3
3	F	0.22	5.3
Mean ± 1 SD		0.25 ± 0.03	5.1 ± 0.6
Normal neonates (3–21 days)			
1 (3 days)	M	0.14	6.2
2 (3 days)	M	0.15	5.4
3 (5 days)	F	0.30	7.6
4 (7 days)	M	0.22	3.9
5 (8 days)	M	0.25	8.6
6 (9 days)	F	0.22	5.9
7 (21 days)	F	0.20	6.9
8 (21 days)	M	0.36	6.7
Mean ± 1 SD		0.23 ± 0.07	6.4 ± 1.3
Infants (2–12 months)			
1 (2 mo)	M	0.39	4.6
2 (4 mo)	M	0.41	6.6
3 (4 mo)	M	0.58	7.1
4 (5 mo)	F	0.46	9.3
5 (8 mo)	F	0.38	6.1
6 (8 mo)	F	0.59	5.5
7 (10 mo)	F	0.35	4.8
8 (12 mo)	M	0.51	8.0
9 (12 mo)	M	0.69	7.0
Mean ± 1 SD		0.48 ± 0.10	6.5 ± 1.4
Normal children			
1 (2 yr)	F	0.48	4.9
2 (4 yr)	M	0.51	6.8
3 (8 yr)	M	0.55	5.7
Mean ± 1 SD		0.51 ± 0.03	5.8 ± 0.8

binding capacity is elevated and it has been estimated that 55 to 60% of the specific binding sites could remain free (10, 31). By contrast, the fetal plasma exhibits low specific binding capacity for cortisol and in addition contains high levels of competing steroids, able to bind to the same sites. For example, 50% of the placental progesterone production goes to the fetus (37), and this steroid has a high affinity for CBG at 37° (16, 34). This situation would result in a relatively large proportion of unbound plasma cortisol in the fetal plasma. The maternal over fetal total plasma cortisol ratio has been evaluated as 3:1, whereas the ratio for the unbound hormone was found to be 1:1 (1). This unbound form may represent the fraction of the hormone exchangeable through the

Fig. 3. Typical graphic representations obtained from the same binding data for plasma from a 4-month-old male infant. The Scatchard plot (a), the Lineweaver-Burk plot (b), and the proportion graph (c) yielded essentially similar results for specific binding capacity (N_s) and apparent association constant (K_d). B and F represent, respectively, the concentration of bound and unbound steroid; T is the total concentration of the steroid present and was used in the proportion graph as described by Baulieu and Raynaud (4).

placental barrier and the available binding sites on the maternal side might contribute to favor the steroid transfer in this direction.

CORTISOL METABOLISM IN PERINATAL PERIOD

After birth, the plasma concentration of steroids of extrafetal origin decreases rapidly, as seen, for instance, with progesterone (11). Neonatal cortisol level in plasma is relatively low (23, 24) and in agreement with the available specific binding capacity given by our data. In fact, the neonatal cortisol half-life is not shorter than in the adult but, on the contrary, rather elevated for large doses (1, 8). These facts cannot be clearly related to the observed increase in plasma CBG during the first year of life and the role (if any) of the increase in plasma cortisol binding sites during this period remains to be elucidated.

EVOLUTION OF CBG LEVEL AFTER BIRTH

CBG synthesis is generally considered to take place in the liver (36). The effect of estrogens on plasma CBG level is well documented, and is believed to reflect increased hepatic CBG biosynthesis (17, 18, 20–22). On the basis of experiments in the rat (17, 18, 36), a release of thyrotropic hormone has been suggested to explain this estrogen effect. To our knowledge, there is no evidence to support the participation of a pituitary factor in the regulation of CBG level in the human. Recently, Beamer *et al.* (5) studied plasma cortisol binding in rhesus monkeys from birth to adulthood and also found low levels in the neonatal period with a progressive increase toward adult values within the first 2 years of life. The time at which adult CBG level was reached coincided with the development of sexual maturity. This is in contrast to our results in the human showing that adult CBG levels are reached much earlier.

The CBG present in the fetal compartment can be supposed to be of fetal origin. However, the time at which the human fetus might be able to carry out the biosynthesis of this glycoprotein is not known. A contribution of the maternal compartment or a possible CBG-like macromolecule of placental origin is purely hypothetical.

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