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THE INFLUENCE OF SERUM PROTEINS ON THE METABOLISM OF CORTISOL BY THE HUMAN PLACENTA

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SUMMARY

The influence of serum proteins on the metabolism of cortisol in various preparations of placental villi was investigated. The rate of metabolism of cortisol to cortisone was compared in several protein-free media, in albumin solutions and in maternal and other sera. Maternal serum retarded metabolism markedly in all instances whereas albumin had only slight effects. The retarding effect of serum was largely abolished by heating to 60°C for 20 min., suggesting that it was due to a heat-labile factor, probably transcortin. Retardation similar to that of maternal serum occurred with serum from a man given high-dose estrogen while less retardation was observed for serum from a normal man. It appears that protein-binding of serum, mainly to transcortin, provides a mechanism for retarding cortisol entry into placental villi, which combined with placental cortisol metabolism prevents excessive maternal cortisol from reaching the fetus.

INTRODUCTION

The role of the high affinity, low capacity indigenous plasma proteins (for which the term "transin" has been suggested by the author [1]) such as the corticosteroid-binding globulin (CBG, transcortin) remains unclear. The relation between the rise of these proteins in pregnancy and hemochorial placentation [2] is of particular interest, since it suggests that there may be some special role for these proteins in this type of placenta. In the present study the conversion of cortisol (F) to cortisone (E) was utilized as a tool for the study of the effects of protein-binding on the uptake of F by the placental villi.

The presence of an 11β-hydroxysteroid dehydrogenase (11β-HSD) in the human placenta was shown by Osinski in 1960 [3] and by Sybulski and Venning in 1961 [4]. However the possible extent of the conversion of F to E was not at first recognized when *in vivo* tracer studies [5] demonstrated the passage of apparently unaltered tracer cortisol from the maternal to fetal circulation. However previous studies [6, 7] had shown large amounts of cortisone in the placenta, and subsequent *in vivo* studies in our own laboratory [8] suggested that most of the maternal cortisol passing across the placenta was converted to cortisone. Using a double tracer constant infusion technique Beitins *et al.* estimated that at term the bulk of fetal cortisone is derived from maternal cortisol [9]. Recently we have provided *in vitro* evidence that about 80% of cortisol is converted to cortisone by the placental villi throughout pregnancy [10].

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MATERIALS AND METHODS

Tissues, including maternal serum, were obtained at deliveries of normal infants, either per vaginam or by Caesarean section, and were refrigerated until used (up to several hours unless otherwise stated).

Cortisol-1,2,6,7-^{3}H(n), S.A. 91 Ci/mmol, cortisone-1,2-^{3}H, S.A. 48 Ci/mmol and dexamethasone-1,2,4(n)-^{3}H, S.A. 21 Ci/mmol, were obtained from the New England Nuclear Corp., Boston, MA. and were repurified using Sephadex LH-20 chromatography as required [11]. Unlabeled steroids were obtained from the Sigma Chemical Co., P.O. Box 14508, St. Louis, MI.

Concentrated human serum albumin (HSA) was obtained through the Canadian Red Cross from Connaught Medical Research Labs., Toronto, Canada.

Pieces of tissue, about 0.5 g, were blotted, weighed, minced coarsely with scissors and added to a measured volume of medium containing tracer ± unlabeled steroid. After incubating at 37°C for 15 min in a shaking Dubnoff bath, the vessels were rapidly cooled in ice water, capped and frozen until analyzed.

For analysis, the steroids were extracted into 5 vol. ethyl acetate. An aliquot of the organic phase was evaporated to dryness under air, dissolved in 0.2 ml methylene dichloride–methanol (98:2, V/V) and applied to automated Sephadex LH-20 columns [12, 13]. Elution was carried out with the same solvent. This procedure separated the F and E fractions so that the radioactivity in each could be determined. Per cent conversion was calculated as $100 \times \text{c.p.m.}_{\text{product}}/\text{c.p.m.}_{(\text{product}+\text{substrate})}$, less the control value calculated the same way. Recovery of radio-

activity as E + F was 80–100% that of the controls which consisted of tracer in medium prepared and incubated along with the tissues. Back reactions were consistently low (usually less than 10%). No activity was demonstrated in maternal serum. Identity of the product as cortisone was established by recrystallization to constant specific activity [13].

Eight flasks of placental cultures (mixed syncitial and cytotrophoblast monolayers, about 1 mg per flask) were kindly prepared by the late Dr. C. J. P. Giroud in the manner which he has described [14] and were incubated without agitation with [^{3}H]-F in 8 ml of various media on the third day. Aliquots of medium were withdrawn at various times and frozen until analyzed as above.

To denature transcortin, serum was heated at 60°C for 20 min [15]. Binding studies were carried out using equilibrium dialysis. To remove endogenous steroids for the tissue culture experiment, 10 ml serum was mixed with 0.3 g charcoal for 30 min at room temperature and then centrifuged until clear; adequacy of removal was verified by radiotransinassay [16]. Transcortin was determined at 4°C using equilibrium dialysis [15].

RESULTS

Placental minces—refrigerated

Protein-free media including Krebs–Ringer bicarbonate-glucose buffer Earle's buffer [18], phosphate buffer 0.1 M, pH 7.4 and 0.9% saline all gave similar results, which were not altered by heating the medium plus tracer to 60°C for 20 min prior to the incubation (Table 1). At both low (no added substrate) and physiological (400 ng/ml) substrate concentrations, the conversion in the presence of albumin 3.4 or 4.3 g/dl tended to be higher than in the absence of protein ($P = 0.05$). Gelatin 0.1 g/dl gave similar results. Pooled maternal serum (F concentration approx. 400 ng/ml) reduced the metabolism of F by 1/3 ($P < 0.001$) while heated maternal serum gave results similar to those obtained for protein-free media. Prior dialysis of maternal serum (heated or unheated) for 17 h at 37°C did not alter the results.

A reciprocal plot of substrate vs velocity (Lineweaver–Burke) gave a K_m of 3 μmol/l and a V_{max} of 270 ng/g/min.

Table 1. Conversion of cortisol to cortisone by human placental minces

Medium				Refrigerated tissue		Fresh tissue	
Composition	Heated	Cortisol (ng/g t.)	Vol med / Vol t.	n	% Conversion (mean ± S.D.)	n	% Conversion (mean ± S.D.)
NS	±	0	4	7	80.3 ± 3.0	4	83.3
NS	±	1600	4	11	66.6 ± 8.4	2	71.1
NS	+	1600*	4	2	71.3		
KRBG	–	0	4			6	77.4 ± 10.5
KRBG	–	1600	4	2	65.1	4	77.9 ± 8.0
PB	–	1600	4	2	68.4		
EB	–	0	4			6	87.6 ± 4.9
EB	–	1600	4			6	77.2 ± 11.7
NS	–	0	4	2	85.0		
NS	–	6400	4	2	43.3		
NS	–	12800	4	2	25.1		
NS	–	25600	4	2	12.2		
NS	–	51200	4	2	5.7		
NS	–	102400	4	2	4.3		
4.3 g/dl alb.	±	0	4	6	85.8 ± 6.6		
4.3 g/dl alb.	+	1600	4	3	75.5		
4.3 g/dl alb.	+	1600*	4	2	83.0		
3.4 g/dl alb.	±	0	4	2	88.2	16	81.7 ± 6.3
3.4 g/dl alb.	±	1600	4	2	85.4	10	82.9 ± 7.3
0.1 g/dl alb.	–	0	4	4	78.6		
0.05 g/dl gel.	–	0	4	4	84.8		
Maternal serum	–	0	4	7	48.2 ± 8.7	17	45.8 ± 10.1
Maternal serum	+	0	4	6	69.7 ± 8.4	17	65.5 ± 11.7
NS	–	3200	8			2	71.1
Maternal serum	–	3200	8			2	42.3
Maternal serum	+	3200	8			2	62.5

0.5 g tissue incubated at 37°C for 15 min
* 1600 ng cortisone also added.
NS = 0.9% saline; KRBG = Krebs–Ringer bicarbonate glucose buffer.
EB = Earle's buffer.
PB = phosphate buffer 0.1 M, pH 7.4.
alb. = albumin; gel. = gelatin; t = tissue; med = medium.

Table 2. Metabolism of cortisol by placental villi (Mean ± S.E.)*

	Refrigerated minces		Fresh minces		Fresh homogenate	
	(% Conversion)	(% PFC†)	(% Conversion)	(% PFC)	(% Conversion)	(% PFC)
Protein-free media	68.2 ± 2.4	100	77.1 ± 2.7	100	86.3	100
Albumin 3.4 g/dl	77.2 ± 4.0	113.2	82.9 ± 3.6	107.5	91.1	105.6
Heated maternal serum	69.7 ± 3.4	102.2	65.5 ± 2.8	85.0	73.3	84.9
Maternal serum	48.2 ± 3.3	70.7	45.8 ± 2.4	59.4	46.5	53.9
Estrogen-treated male serum			37.5	48.6		
Normal male serum			55.9	72.4		

* Substrate concentration 800 ng/0.5 g tissue in 4 vol. medium incubated 15 min. at 37°C. Where S.E.'s are not indicated, the mean of duplicate values is given.
† PFC = protein-free control

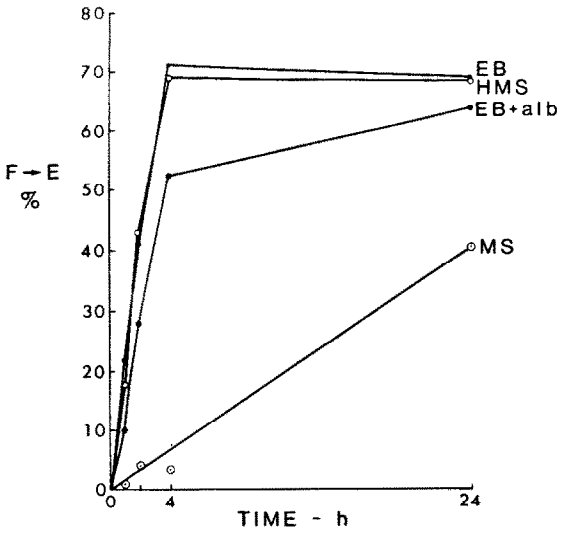

Fig. 1. Metabolism of [^{3}H]-cortisol by placental cells in monolayer culture. Each value is the mean of duplicates. Media included EB (Earle's buffer), HMS (charcoal-treated maternal serum diluted 1/4 with Earle's buffer and heated at 60°C for 30 min.), EB + alb (3.4 g/dl human albumin in Earle's buffer), and MS (charcoal-treated maternal serum diluted 1/4 with Earle's buffer). Cells, 1 mg were incubated in a starting volume of 8.0 ml containing 2.0 ng [^{3}H]-cortisol for 24 h at 37°C. Samples of 0.5 ml of medium were withdrawn at intervals.

Placental minces—fresh

Since the early experiments were done several h after obtaining the tissue and without aerating the solutions, it seemed likely that many or most of the cells were dead and that these would not reflect the *in vivo* situation. Therefore experiments were carried out in which the placenta was obtained and incubated immediately after delivery by Caesarean section. The solutions were gassed with 95% O_2/5% CO_2 and the incubation was completed within 30 to 45 min of delivery. The results are shown in Table 1 and are summarized and compared with those of male sera and refrigerated minces in Table 2. Only the results in protein-free media differed, being slightly higher ($P < 0.05$) for the fresh tissue. Albumin did not increase the metabolism in fresh tissue ($P > 0.05$). Maternal serum again reduced the metabolism markedly ($P < 0.001$) while heated maternal serum reduced it slightly ($P < 0.05$). Serum from an estrogen-treated man (ethinyl estradiol 0.3 mg daily) retarded conversion even more strongly than maternal serum while that from a normal man had a lesser effect than either, effects in proportion to their transcortin concentrations (2.0, 1.6 and 0.8 μM respectively).

Fresh homogenates

A single study in duplicate was made of fresh placenta which after mincing was homogenized for 1 min using an all-glass homogenizer. The results were similar to those for minces, though tending to be slightly higher (Table 2).

Cultured placental cells.

Since mincing produces many broken cells and these might influence the results disproportionately, placental cultures were also investigated. Here the integrity of the cells was assured by direct observation. The amount of tissue (1 mg) was very small by comparison to the amount of protein in the 8 ml of medium so that the relative concentrations of enzyme and binding-protein were greatly different from the minces. In this study shown in Fig. 1 and Table 3, the metabolism was retarded 90% in the presence of maternal serum diluted 1/4, but not by the same diluted serum heated to 60°C for 20 min. Albumin 3.4 g/dl retarded the metabolism by 25%. This system was thus more sensitive in showing the effects of protein-binding. The rate of metabolism was almost linear until it plateaued at about 70%. The small amount of cells (1 mg) converted 70% of the 2.0 ng F present by 4 h. This activity in protein-free medium (8 ng/g/min) was less than that observed for minces, probably because of the lack of agitation during the incubation. Despite the relatively large amount of F bound to albumin (40%) the influence of albumin binding on metabolism was relatively weak compared to that of unheated serum, presumably due to transcortin.

Table 3. Influence of binding on metabolism of cultured placental cells

	Cortisol concn. (ng/ml)	Observed Control 4 h	Inhibition of metabolism (%)	Cortisol bound† (%)
Protein-free (EB)	0.25	100.0	0.0	0.0
Maternal serum diluted 1/4 (EB)	0.25	9.1 9.7	90.5	84.9
Heated maternal serum diluted 1/4 (EB)*	0.25	99.4 94.1	3.3	13.3
Albumin 3.4 g/dl (EB)	0.25	75.8 74.6	24.8	41.2

* Albumin concn. 0.9 g/dl.
† Mean of duplicate values.
(EB) = Earle's buffer.

Table 4. Distribution of radioactivity in placental tissue and medium*

Tracer	Duration of incubation (min)	Non-radioactive steroid added	c.p.m.$_{0.5g\,tissue}$/c.p.m.$_{0.5ml\,medium}$		
			Saline	Maternal serum	Heated maternal serum
E	15	—	1.90	0.82	—
E	15	1600 ng E	1.65	0.91	—
F	15	—	1.95	0.58	—
F	15	1600 ng F	1.90	0.82	—
E	120	—	1.67	0.70	0.65
E	120	5000 ng E	1.67	0.84	0.69
F	120	—	1.63	0.65	0.65
F	120	5000 ng F	1.63	0.75	0.79
Dex	120	—	2.63	0.79	0.87
Dex	120	5000 ng Dex	2.45	0.79	0.67

* 0.5 g tissue incubated in 2 ml medium, mean of duplicate values.

Binding to placental protein.

Another factor which could alter the expected effects of protein-binding would be the presence of binding proteins within the cell. To explore this possibility placental minces were incubated as before with either tracer E or F or dexamethasone, a synthetic analog of F which is not bound by transcortin. After separating the tissue from the medium, the steroids of both were extracted into methylene chloride which was then assayed for total radioactivity. The results are shown in Table 4. When 0.9% saline was used as the medium the cpm in the tissue starting with either tracer E or F were approximately double those of the medium, suggesting that there is binding to placental cells. For maternal serum the cpm were higher in the medium and heating made little difference. Addition of relatively large amounts of F or E also made little difference, probably because of the rapid conversion of F to E and the weaker binding of E to transcortin. Thus most of the binding in serum was probably that of E attributable to albumin which is unaffected by heating to 60°C. Results at 15 and 120 min were much the same, the one low value of 0.58 for maternal serum probably being due to incomplete conversion of F to E by 15 min and therefore increased binding of F to transcortin. No high affinity binding was demonstrated in the tissue. Dexamethasone was taken up by the cells more avidly than F or E but there was little change on adding large amounts of unlabeled steroid, suggesting that no high-affinity, low capacity binding site was present, although such a site has not been excluded by this study.

DISCUSSION

It has been suggested by O'Hare[17] that *in vitro* studies using homogenates, minces and cell suspensions may reflect only the activity of damaged cells and he preferred to use cell cultures. However the latter cannot be grown in exactly the same hormonal milieu as the parent cells, a factor which also may produce unphysiological results. In this study placental cells grown in culture gave qualitatively similar results to those using fresh minces.

The similarity of the results among all the preparations suggest that this enzyme is very stable. At low substrate concentrations (no substrate added) placental minces converted up to about 90% of the substrate. At higher physiological concentrations (1600 and 3200 ng/g) conversion was 70 to 80%.

Under the conditions used here the influence of albumin was barely demonstrable, purified albumin tending to increase metabolism while heated serum, in which transcortin had been denatured, decreased it slightly. However both of these effects might be artifactitious, the effect of purified albumin (from non-pregnant sources) being brought about by removal of an inhibitor diffusing from the placental cells, that of heated maternal serum being due to incomplete denaturation of transcortin by heating, likely to occur in the presence of large amounts of cortisol [15]. Since the concentration of F and E in placental cells was almost twice that of the saline medium and about 0.7 that of heated serum it seems likely that there is a high capacity, low affinity binding protein in the cytosol of placenta cells which is comparable to albumin. If steroids cross cell membranes freely, the presence of such a protein might also be expected to diminish the apparent effects of albumin on entry and metabolism. The most accurate reflection of the effect of albumin may be that of the placental cells in culture where the amount of an inhibitor would be very low relative to the amount of protein in the medium and where denaturation of transcortin in the charcoal-treated maternal serum could be expected to be complete [15]. In this situation the effect of albumin was to retard metabolism weakly.

Although it is 20 years since the discovery of the high affinity, low capacity protein which binds cortisol by Daughaday[18] and by Sandberg and Slaunwhite[19], the fundamental role of transcortin and of the other transins remains obscure. Whether they are synthesized in tissues other than the liver or whether they may enter the cells of certain tissues is still not clear. That they retard metabolism in at

least some tissues is apparent from much previous work [20–25] and from the present study. That the interaction with albumin may be important for the action of aldosterone has been suggested by Funder *et al.*[26] and for that of estrogens by Burke and Anderson[27]. A possible direct action of the sex hormone binding globulin on prostatic metabolism has been suggested by Mercier-Bodard *et al.*[28].

Seal and Doe[2] have observed that although unbound cortisol rises in many mammalian species during pregnancy, only in animals with a hemochorial placentation does transcortin also rise during pregnancy. With this type of placentation, maternal blood comes into direct contact with the cells of the placental villi whereas in the other types there is separation by one of more maternal cell layers. While the hemochorial type has the advantage of increased diffusion there is also a greater risk that excessive amounts of undesirable substances may enter the fetal circulation. Excessive cortisol reaching the fetus in early pregnancy might be expected to retard growth and to suppress the fetal pituitary-adrenal axis. At least 3 mechanisms appear to contribute to preventing this—the ability of many fetal tissues to inactivate cortisol to cortisone [10], that of the placental villi to do the same, and the increased concentration of transcortin in the maternal circulation.

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