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PLASMA C-21 STEROIDS IN CONSCIOUS PREGNANT AND NON-PREGNANT RABBITS WITH CHRONIC CATHETERIZATION OF THE FEMORAL ARTERY AND THE PORTAL AND HEPATIC VEINS

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Summary—In conscious non-pregnant ($n = 8$) and pregnant ($n = 7$) rabbits, blood samples were collected by chronic catheterization of the femoral artery and the hepatic and portal veins. In the non-pregnant group, deoxycorticosterone (DOC), corticosterone (B), cortisone (E) and cortisol (F) were determined by RIA. In the pregnant group, progesterone (P) and 20α-dihydroprogesterone were also radioimmunoassayed. The arterio-venous difference of the levels observed has demonstrated the role of the liver and the splanchnic area in steroid metabolism. Moreover, the comparison of the steroid pattern in the two groups showed that gravidity was characterized by a marked increase of F and E but not of B and DOC levels. Thus, the ratio of F/B in the femoral artery was markedly increased in the pregnant animals; this ratio ranged from 0.20 to 1.12 in the non-pregnant group, and from 1.3 to 12.5 in the pregnant group.

INTRODUCTION

Although the patterns of either ovarian [1–4] or adrenal steroids [5, 6] have been described in the peripheral plasma of either pregnant or non-pregnant rabbits, few studies have been concerned with both groups of steroids [7, 8]. In addition, none of these studies involved deoxycorticosterone (DOC) evaluation despite the fact that, in addition to its adrenal production, DOC is also formed in the liver [9, 10] and in the spleen [11] from progesterone (P), which has been demonstrated to increase during gestation [1–4, 7, 8]. Moreover, hepatic C-21 hydroxylation of P has been shown to be involved [9, 10] in the oxidative pathway of its metabolism in the rabbit [12–14]. Furthermore, blood samples were collected from either anesthetized [1, 3] or conscious animals [4–8] and sampling was performed either from an ear vein [5, 6, 8] or by cardiac puncture [1, 3–5, 7]. Since anesthesia may influence peripheral plasma levels of steroids [15], blood sampling in conscious animals under unstressed conditions would provide a better insight into the hormone milieu.

The aim of the present study was to investigate the role of the liver and the splanchic area in steroid metabolism in conscious pregnant (day 29) and non-pregnant rabbits with chronic catheterization of the femoral artery and the hepatic and portal veins. In the blood sampled from these three sites, DOC, corticosterone (B), cortisone (E) and cortisol (F) were determined in the pregnant as well as in the non-pregnant rabbits. In addition, P and 20α-dihydroprogesterone (DHP) were also estimated in the pregnant animals. In view of the arterio-venous difference in steroid levels the role of the liver and the splanchic area in steroid metabolism under steady-state conditions could thus be studied in both groups of animals.

EXPERIMENTAL

Animals and surgical procedure

Experiments were carried out on 8 non-pregnant and 7 pregnant rabbits (New Zealand) weighing 3.5–4.0 kg. Animals were housed in individual cages at 19°C with light from 8.00 a.m. to 10.00 p.m. They were fed laboratory chow *ad libitum* (16% protein, 15% carbohydrate, 3% fat and 14% fiber). Mating was performed in the laboratory and the day of mating was taken as day 0 of gestation. The number of fetuses per litter was 2–10.

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Surgery was performed under general anesthesia (ketamine hydrochloride, 35 mg/kg body wt and xylazine hydrochloride, 8 mg/kg body wt). Three polyvinyl catheters (i.d. 0.4 mm; o.d. 0.8 mm) were implanted into the right hepatic vein, the portal vein and the femoral artery, respectively, as described previously [16]. Upon completion of the surgery, the catheters were passed s.c. and exteriorized at the back of the neck and secured in a cap. The catheters were filled with heparin solution (400 U/ml). Food intake was monitored before and after surgery until it was reestablished (2–3 days).

Methods

The technique was designed so that all the steroids could be determined in the same plasma sample by a combination of methods already described for the determination of P and DOC [17] and corticosteroids [18] in humans.

In addition to the chemicals and reagents already described [17–20], ^{125}I labeled P (SA 2000 Ci/mmol) and tritiated DHP were purchased from Amersham France (Les Ulis, France) and the antiserum anti-DHP from Endocrine Sciences (Tarzana, CA, U.S.A.). The antiserum was raised in rabbits injected with 20α-DHP-3-oxime-BSA and was used at a 1:24,000 dilution.

A brief description of the procedure used follows. Approx. 1000 cpm of tritiated P, B, E and F were added to 0.2–0.5 ml plasma to monitor methodological losses. Extraction was performed with 5 ml methylene chloride. The solvent was evaporated to dryness and the residues redissolved with 2 × 0.2 ml methylene chloride and applied on columns (8.5 × 170 mm; 10 ml disposable pipettes Volac) of Sephadex LH-20 swollen in methylene chloride [18]. After a 4 ml wash, P, DOC and DHP were eluted together by 5 ml methylene chloride. The next 5 ml eluted B. The following 4 ml were discarded and E was eluted with 10 ml. Then 4 ml were discarded and F was eluted with 16 ml. All eluates were evaporated to dryness under a stream of nitrogen. The three fractions corresponding to B, E and F were redissolved in 1 ml of either ethanol (B) or phosphate buffer (E and F) and aliquots were pipetted for estimation of recovery or RIA [18].

The first fraction (P, DOC and DHP) was submitted to a second column chromatography. The residue was redissolved in 0.5 ml isooctane and applied to Celite columns as already described [17]. Elution was carried out stepwise. The first fraction of 7 ml isooctane was discarded. P was eluted with the next 8 ml. The following three fractions—10 ml of isooctane–toluene (90:10, v/v), 12 ml isooctane–toluene (80:20, v/v) and 2 ml isooctane–toluene (60:40, v/v)—were discarded. Finally, DOC and DHP were eluted together in the same 10 ml of the last solvent mixture. The eluates corresponding to P, DOC and DHP were evaporated to dryness, and redissolved in 1 and 2 ml ethanol, respectively. 0.5 ml of the P eluate was pipetted for the estimation of the recovery and 0.4 or 0.5 ml for RIA as described [17] except that the antiserum was used at a 1:300,000 dilution and the tracer was labeled with ^{125}I.

Similarly, samples from the other eluate (DOC and DHP) were pipetted for RIA [17]. In the case of these last two steroids, methodological losses were evaluated by calculating the recovery of either tritiated DOC or DHP added to two plasma pool aliquots and submitted to the whole procedure.

The accuracy was assessed by the evaluation of the recovery of the tritiated steroids added to 6 plasma samples and processed through the whole procedure. The results are given in Table 1. Intra- and inter-assay variabilities were evaluated by the coefficient of variation (CV) of the results obtained when the same plasma pool samples were determined in the same or in different series of assays. The data obtained for each steroid are reported in Table 1. As shown, the technique was highly reproducible whatever the steroid considered.

The specificity, as assessed by the cross-reactivity of the antisera, has already been reported for P, DOC [17], B, E and F [18, 20]. It was also shown that column chromatography, performed according to the precedure outlined above, achieved a good separation of any of the determined steroids from those displaying important cross-reaction with the corresponding antisera. For instance, although the cross-reaction of 5α- and 5β-pregnanedione with P-antiserum was 15.2 and 61.1%, respectively these steroids did not interfere with P assay since they were not eluted in the same fraction from the Celite column [17]. Concerning DOC and DHP, although they were eluted in the same fraction, they were not liable to interfere with each other in the assay because their cross-reaction with the other antiserum was not significant. In fact, the cross-reaction of DOC with DHP-antiserum was $< 0.02\%$ and that of DHP with DOC-antiserum was $< 0.10\%$.

Table 1. Evaluation of accuracy and intra- and inter-assay variabilities

Steroid	Mean recovery (and range) of tritiated steroid (%)	Intra-assay variability				Inter-assay variability			
		n	Mean	SD	CV (%)	*n*	Mean	SD	CV (%)
P(ng/ml)	79.2	9	0.5	0.04	8.1				
	(77.3–82.4)	9	2.1	0.09	4.6	6	2.1	0.29	13.9
DHP(ng/ml)	71.8	8	0.5	0.06	12.2				
	(68.8–76.1)	7	9.9	0.39	4.0	6	9.7	1.03	9.9
DOC(pg/ml)	73.9	7	196	16	8.4				
	(68.5–77.8)	8	476	29	6.1	6	520	51	9.9
B(ng/ml)	75.0	11	1.8	0.09	4.9				
	(73.0–80.2)	10	11.3	0.62	5.5	5	10.4	1.26	12.1
E(ng/ml)	76.5	10	1.8	0.10	5.4				
	(72.1–80.2)	10	9.8	0.58	5.9	6	9.1	1.14	12.7
F(ng/ml)	79.2	11	9.7	0.48	5.0	6	9.3	1.20	12.4
	(70.6–85.7)	11	50.3	1.56	3.1				

Statistical analysis

Results were expressed as the arithmetic mean ± SD. After checking that the data were sampled from a Gaussian population, by the Kolmogorov–Smirnov test, the group means were compared by analysis of variance and *t*-test. When the variation could be assumed to be unidirectional, one-tailed tests were applied for group comparisons.

RESULTS

Non-pregnant rabbits

Steroid levels determined in the blood plasma, sampled in the femoral artery and the portal and hepatic veins of the 8 non-pregnant rabbits, are listed in Table 2.

In the femoral artery and the portal vein mean B levels were the most important, whereas in the hepatic vein F levels exceeded those of B. Thus, the mean ratio of F/B was significantly higher ($P = 0.02$) in the hepatic vein than in the other sites (Table 3). Similarly, F always exceeded E, but the mean ratio F/E was lower in the portal vein than in either the femoral artery ($P = 0.02$) or the hepatic vein ($P = 0.01$). No significant difference could be demonstrated between the mean F/E ratios in the femoral artery and the hepatic vein.

When the steroid levels were considered according to the site of blood sampling, it was found that those observed in the femoral artery and in the portal vein were similar. However, the levels observed in the hepatic vein were significantly lower than those obtained in the two other sites: $P = 0.004$, 0.005, 0.001 and 0.02 for DOC, B, E and F, respectively.

Pregnant rabbits

Listed in Table 3 are the plasma steroid levels observed in the 7 pregnant rabbits. F levels were the highest, while those of DOC were the lowest in the blood sampling sites.

The mean steroid concentrations, except P, were comparable in the femoral artery and the portal vein. P levels were significantly higher in the femoral artery than in the portal vein ($P = 0.04$). The mean levels observed in the hepatic vein were always lower than those found in the femoral artery: $P = 0.01$ for P, DHP and E; and $P = 0.0005$, 0.04, 0.009 for DOC, B and F, respectively (Table 4).

The mean ratio of F/E levels in the femoral artery was significantly higher ($P = 0.04$) than in the portal vein and significantly lower ($P = 0.002$) than in the hepatic vein (Table 5).

No difference could be found between the mean values for the ratio of F/B in the femoral artery and the portal vein. Conversely, the mean ratio obtained in the hepatic vein was significantly higher than that either in the femoral artery or the portal vein ($P = 0.02$) (Table 5).

Table 2. Plasma steroid levels in the non-pregnant rabbits ($n = 8$)

Steroid	Site of blood sampling		
	Femoral artery	Portal vein	Hepatic vein
DOC (pg/ml)	259 ± 168[a]	234 ± 232	56 ± 56
	(102–621)	(107–802)	(15–179)
B (ng/ml)	10.7 ± 9.3	9.8 ± 8.7	0.6 ± 0.7
	(3.1–28.7)	(2.4–26.7)	(0.1–1.9)
E (ng/ml)	0.8 ± 0.4	1.1 ± 0.4	0.3 ± 0.2
	(0.3–1.1)	(0.4–1.7)	(0.1–0.6)
F (ng/ml)	3.9 ± 2.0	3.5 ± 1.4	1.9 ± 1.0
	(0.7–7.4)	(0.8–5.5)	(0.3–2.9)

[a]Mean ± SD (and range).

Table 3. Ratio of plasma F levels to those of E and B in the non-pregnant rabbits ($n = 8$)

Ratio	Site of blood sampling		
	Femoral artery	Portal vein	Hepatic vein
F/E	4.7 ± 2.1[a]	3.5 ± 1.5	6.1 ± 1.5
	(2.2–9.4)	(1.1–5.9)	(3.7–8.3)
F/B	0.53 ± 0.36	0.67 ± 0.56	6.8 ± 5.9
	(0.20–1.12)	(0.13–1.70)	(0.8–14.5)

[a]Mean ± SD (and range).

Table 4. Plasma steroid levels in the pregnant rabbits ($n = 7$) on the 29th day of gestation

Steroid	Site of blood sampling		
	Femoral artery	Portal vein	Hepatic vein
P (ng/ml)	3.8 ± 3.2[a] (0.5–9.8)	2.4 ± 2.0 (0.7–6.4)	0.3 ± 0.08 (0.2–0.4)
DHP (ng/ml)	2.5 ± 1.9 (0.5–6.1)	1.9 ± 1.5 (0.2–4.1)	0.8 ± 0.4 (0.2–1.5)
DOC (pg/ml)	405 ± 134 (230–593)	347 ± 170 (130–650)	235 ± 82 (140–364)
B (ng/ml)	8.9 ± 5.7 (1.8–18.3)	8.2 ± 4.3 (2.0–14.3)	4.0 ± 2.6 (2.0–6.7)
E (ng/ml)	6.0 ± 4.5 (1.9–12.0)	7.0 ± 4.9 (2.8–15.2)	2.6 ± 2.4 (0.6–6.2)
F (ng/ml)	41.7 ± 30.9 (13.4–92.6)	41.3 ± 29.6 (12.5–84.5)	33.5 ± 26.2 (8.3–64.8)

[a]Mean ± SD (and range).

Comparison of the steroid pattern between pregnant and non-pregnant rabbits

When the steroid pattern of the two groups was compared, it could be demonstrated that gestation appeared to be characterized by a marked increase of F ($P = 0.002$) and E ($P = 0.01$) levels, while no statisically significant difference could be demonstrated for B and DOC. Thus, the mean ratio of F/B observed either in the femoral artery or in the portal vein was higher ($P = 0.0004$) in pregnant than in non-pregnant rabbits. The difference was also significant for the ratio found in the hepatic vein ($P = 0.004$).

The mean value of the ratio F/E in the three sites was also significantly higher in the pregnant than in the non-pregnant rabbits ($P = 0.005$).

DISCUSSION

It may be assumed that there is no significant difference in the steroid levels observed in the blood sampled from any artery. Thus, the steroid concentrations entering the liver via the hepatic artery may be regarded as equivalent to those found in the femoral artery.

Steroid levels in the non-pregnant rabbits

In non-pregnant rabbits, F levels were not very different from those observed by others [5, 21] and obtained by chromatographic techniques, but they were markedly lower than those found by either a direct RIA [8] or a fluorometric technique despite a preliminary chromatographic purification [22]. Thus, the specificity of the techniques used may account, at least in part, for these differences. The present B and particularly, E levels were surprisingly lower than literature data [5, 21, 22], though a chromatographic step was included in the techniques used. Since the number of the animals studied was rather limited, inter-individual variability may explain this variance. The DOC levels reported here seem to be the first of this steroid in rabbit blood.

The fact that B levels always exceeded those of F is consistent with the literature data [5, 21, 22], yet the ratio of F/B observed here is only comparable with that reported by Ganjam *et al.* [22].

The comparison of the steroid levels in the three sampling sites has clearly demonstrated that there is an important metabolism of these steroids in the liver. Moreover, the hepatic uptake of B seems to be the most important.

Table 5. Ratio of plasma F levels to those of E and B in the pregnant rabbits ($n = 7$) on the 29th day of gestation

Ratio	Site of blood sampling		
	Femoral artery	Portal vein	Hepatic vein
F/E	9.0 ± 2.6[a] (5.6–12.6)	7.4 ± 2.7 (4.3–12.4)	21.9 ± 7.1 (13.7–31.6)
F/B	7.4 ± 4.4 (1.3–12.5)	8.2 ± 6.9 (1.8–15.6)	11.7 ± 4.9 (5.4–20.2)

[a]Mean ± SD (and range).

Pregnant rabbits

The present P levels were similar to those found by Browning *et al.* [2] but lower than those reported either at 29 days of gestation [1, 3, 4, 7] or in the 4th week of gestation [8]. The high levels obtained in those studies might be related to the specificity of the assay used in at least two of them, where P was determined by non-chromatographic RIA [4, 8]. However, in the other studies [1, 3, 7] a chromatographic step was included so that the specificity of these techniques does not appear to be questionable. In addition, blood sampling conditions could not explain these differences since, apart from Lau *et al.* [3], all the other authors collected blood from conscious animals. However, inter-individual variability, since the number of determinations was rather limited in either their series or ours, as well as the pulsatile patterns demonstrated for ovarian steroids in pseudopregnancy [23], might account for these variations.

The concentrations of DHP were comparable with Browning *et al.* [2] data but markedly lower than those found by others [3]. Here again this difference may be attributed to inter-individual variability as well as to the pulsatile pattern [23]. In addition, DHP was generally lower than P, a finding also reported by Browning *et al.* [2].

To our knowledge, the present data concerning DOC levels in the pregnant rabbit are reported for the first time.

F and B levels were comparable with the data reported for the same day of gestation by Baldwin and Stabenfeldt [7]. Similarly, the F levels were consistent with the findings of Kriesten and Murawski [8] during the 4th week of gestation and very similar to those observed by Barr *et al.* [6] at 29 days, despite the fact that the methodology used by the two groups was different. Indeed, F was assayed directly in one case [8] and after TLC in the other [6]. However, the present F levels were higher than those found by Mulay *et al.* [5].

E levels were rather lower than the Barr *et al.* [6] data and the reason for such variance is not clear.

In comparison with F levels those of E were always lower in the femoral artery and this is consistent with the Barr *et al.* [6] data at the end of gestation. Indeed, while E exceeded F levels in peripheral plasma until the 25th day of gestation the latter became prevalent thereafter [6]. This might be due to the increase of the corticosteroid-binding protein during gestation, which has a higher binding capacity for F than for E [24].

Similarly, F levels markedly exceeded those of B and this agrees with the Baldwin and Stabenfeldt [7] data at the same age of gestation. An increase of F throughout pregnancy was also reported by Mulay *et al.* [5] but the F/B ratio was much lower than that found here. The fact that a shift in corticosteroid production occurs during pregnancy may be compared with the effect of prolonged ACTH stimulation of the adrenal in the rabbit [21, 25]. Indeed, this stimulation was shown to enhance the production of 17α-hydroxylated steroids, while that of B was decreased [21]. Whether such an ACTH stimulation of the adrenals occurs in the rabbit during gestation remains to be demonstrated. Moreover, as pointed out above, the increase of the corticosteroid-binding protein with a binding capacity greater for F than for B [24] might also amplify the phenomenon.

The finding of lower levels in the hepatic vein than in the femoral artery and the portal vein suggests a hepatic uptake of all these steroids and their subsequent metabolism, as was observed in the non-pregnant group.

P seems to be metabolized in the liver and to a lesser degree in the splanchnic area, since its levels were also lower in the portal vein than in the femoral artery. Such a metabolism of P in the splanchnic area and the liver was recently shown in the rabbit. Indeed, when tritiated P was given by either the i.p. or the i.v. route, unmodified peripheral plasma P was substantially lower following i.p. than i.v. administration [26].

In comparison with other steroids, the finding of a relatively less important decrease of F levels between the hepatic vein and the femoral artery might be related to the fact that F is predominantly bound to CBG [24]. Moreover, the 11β-dehydrogenase activity responsible for the conversion of E to F might be important in the liver, as was demonstrated in fetal rabbit lung [27] and in human fetal and adult liver [28]. This conversion would thus reduce the F decrease as a result of its metabolism in the liver.

E concentrations were generally highest in the portal vein though the difference was not significant in comparison with the femoral artery. This finding might suggest that F was converted to E in the splanchic area. Such a conversion in the stomach, pancreas, intestine and spleen of the human fetus has already been demonstrated [28].

Although not significant, the lower mean levels of DOC in the portal vein than in the femoral artery do not seem to agree with the recent *in vitro* finding of P 21-hydroxylase activity in the spleen of the rabbit [11]. In addition, the significantly lower DOC concentrations in the hepatic vein in comparison with the femoral artery are not in variance with the *in vitro* demonstration of a high P 21-hydroxylase activity in the microsomes and mitochondria of the rabbit liver [10] since DOC undergoes further metabolism *in situ*. In fact, DOC is an intermediate in the oxidative pathway of P metabolism in the liver [9].

Comparison of steroid pattern in the two animal groups

When the steroid pattern observed in the non-pregnant animals is compared with that in the pregnant animals, a marked increase of all steroids except B may be noted. The finding of comparable B levels in the two groups of rabbits is consistent with the results of Mulay *et al.* [5]. Similarly, the increase during gestation of P and F confirms other data [5, 8, 23].

In conclusion, although this study was limited to a small number of animals and to only 1 day of gestation, it has provided valuable information on steroid levels in non-pregnant as

well as pregnant rabbits under steady-state conditions. Moreover, the levels observed at the three sites of blood sampling of the different steroids have stressed the role of the liver in steroid metabolism and in the conversion of E to F. In addition, DOC levels in circulating plasma have been reported here for the first time in both non-pregnant and pregnant rabbits.

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