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EFFECT OF PHOSPHOLIPIDS ON THE SUBCELLULAR DISTRIBUTION OF PROGESTERONE IN PREGNANT RAT MYOMETRIUM AND ON PROTEIN BINDING OF PROGESTERONE

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SUMMARY

Treatment of pregnant rats with a fat emulsion from soya beans, which contains lecithin and which increases the uterine activity in pregnant rabbits and women, changed the subcellular distribution of labelled progesterone in the myometrium and increased the amount of unconjugated metabolites of progesterone. Oestrogen pretreatment increased the uptake of radioactive progesterone in the myometrium. In equilibrium dialysis experiments this lecithin fraction was able to release radioactive progesterone from the binding proteins in human plasma and in pregnant rat myometrium, the radioactive progesterone concentrating in the outer phase.

INTRODUCTION

It has been demonstrated that after infusion of an intravenous fat emulsion (Infonutrol), pregnancy can be interrupted in rabbits by giving oxytocin[1,2]. The active agent in the fat emulsion proved to be the phospholipid component of the solution. It was further found that administration of exogenous progesterone abolished the effect of the phospholipid, pregnancy being maintained, although the same dose of phospholipid and oxytocin without the progesterone treatment resulted in premature labour. Infusion of an intravenous fat emulsion containing the same phospholipid fraction in patients admitted for legal abortion at 9–15 weeks of pregnancy increased the myometrial activity in eight patients out of eleven[3]. Pregnanediol excretion was increased after the treatment, but there were no significant changes in the oestriol excretion or plasma progesterone level. When this treatment was given at term to 13 patients, the ensuing progressive contractions led to normal vaginal delivery in 8[4]. In three patients labour had to be terminated by Caesarean section.

In these investigations it was demonstrated that infusion of the phospholipid increased uterine activity; however, the mode of action remained obscure. In the experiments with rabbits it seemed that the infusion counteracted the effect of progesterone on the myometrium.

In studies on the metabolism and subcellular distribution of progesterone in the myometrium of the pregnant rat, Wichmann[5] demonstrated that unchanged progesterone was retained for long periods in the myometrium. The reduction products of progesterone and 17α-hydroxy-progesterone were not retained by the myometrium. The effect was pronounced in the heavy microsomal fractions. This intracellular distribution of progesterone was altered around the time of the delivery, indicating that the intracellular distribution of progesterone might have some physiological significance[6].

Whether the increased myometrial activity induced by phospholipid infusion is based on altered distribution of progesterone in the target tissue, myometrium, could be studied by determining the effect of the phospholipid on the intracellular distribution of progesterone in the myometrium. The ability of the myometrium of the pregnant rat to retain progesterone but not its metabolites indicates specific protein binding. Therefore, it also seemed important to study the effect of the phospholipid fraction on the binding of progesterone by specific proteins.

This study reports the effect of the phospholipid on the distribution of progesterone in the myometrium of the pregnant rat and its effect on the binding of progesterone by proteins.

MATERIALS AND METHODS

Subcellular distribution of progesterone in myometrium

Female rats of the Sprague–Dawley strain, 20–21 days pregnant, were used as test animals. The rats were anaesthetized with pentobarbital and received an intra-aortic injection of [4-^{14}C]-progesterone, S.A. 46 mCi/mmol. 2 μCi was given per 250 g of body weight and the animals were bled after 1 h.

There were four groups, each consisting of six animals. The first, or control, group received radioactive progesterone only. The second group received an injection of 2 mg oestradiol the day before administration of the labelled progesterone. The third group received, in addition, an intra-arterial injection of a fat emulsion, Infonutrol (Astra A.B., Sweden) half an hour before administration of [^{14}C]-progesterone. The amount of Infonutrol was 2 ml. The fourth group was first injected with radioactive progesterone and half an hour later with 2 ml of Infonutrol. In all groups the animals were killed one hour after administration of the progesterone.

After the animals had been exsanguinated, the uterus was dissected free of adjacent tissue, the foetuses and placentas were removed, and the endometrium was scraped off with a scalpel. The myometrium (3 g wet weight) was placed in ice-cold sucrose solution (0·25 M and buffered to pH 7·4 with 1 M Tris buffer). The tissue was minced with scissors and the suspension was then homogenized in a Potter homogenizer. The homogenate was divided into subcellular fractions by centrifugation. The fractions obtained were as follows: the nuclear-myofibrillar fraction was sedimented at 800 *g* for 10 min, the mitochondrial fraction at 10,000 *g* for 15 min from the supernatant of the preceding centrifugation, the heavier microsome fraction was sedimented at 20,000 *g* for 15 min and the light microsomes at 105,000 *g* for 1 h. Finally the supernatant was collected. All particulate fractions were washed and recentrifuged twice. The homogeneity of the fractions was checked by means of electron microscopy. Nitrogen was determined by Kjeldahl's micromethod[7]. The radioactive steroids were extracted as follows: the subcellular fractions were extracted three times with 70% methanol solution, then twice with ethyl acetate. The combined extracts were evaporated under reduced pressure to aqueous volume and extracted three times with ethyl ether. The combined ether extracts were evaporated and the residue dissolved in 70% methanol and kept in the cold (−18°C) for 18 h to allow the neutral fats to precipitate. The fats were sedimented at 3000 rev/min for 10 min and the supernatant evaporated to dryness under nitrogen. The residue was dissolved in ethyl acetate. One fifth of the solution was saved for direct counting and the remaining

four fifths subjected to thin-layer chromatography after addition of the carrier progesterone.

In thin-layer chromatography the solvent system chloroform-acetone (90:10, v/v) was used. After development of the chromatoplate, the area corresponding to the R_f limits of progesterone was cut out, and the progesterone eluted and counted in a Packard Liquid Scintillator Counter, using 0·01% 4-bis-2(5-phenyl oxazolyl)-benzene (POPOP) mixed with 0·4% 2·5-diphenyloxazole (POP) in toluene. The identity of the progesterone was verified by the use of a reference standard in thin layer chromatography and also by gas–liquid chromatography of the non-radioactive carrier on three different liquid phases: 3% F-60 (methyl-*p*-chlorophenyl siloxane polymer), 1% Z (co-polymer made from ethylene glycol, succinic acid and a methyl siloxane monomer) and 1% XE-60 (cyanoethyl silicone).

Studies on binding of progesterone by proteins

In the *in vitro* experiments the active component of Infonutrol, purified lecithin SA in the aqueous emulsion, replaced the intravenous fat emulsion used *in vivo*. The biological materials studied were 1% plasma solution and the homogenate of pregnant rat myometrium at term. The plasma was obtained from a woman taking oral contraceptives and having plasma with a high progesterone-binding capacity. The uterus of pregnant rats was freed from the adjacent tissue, foetuses, placentas and endometrium. Then the myometrium was transferred to ice-cold 0·025 M Tris buffer in 0·45% NaCl and homogenized with a Potter homogenizer to achieve 10% homogenate (wet weight/volume), which was diluted to a protein concentration of 1 mg/ml according to protein determination [8].

The progesterone-binding capacity of the samples and the effect of lecithin on the binding of progesterone by proteins were determined by equilibrium dialysis [9]. The dialysis system consisted of 1·5 ml of 1% plasma solution or rat myometrial homogenate as the inner phase and 15 ml of 0·025 M Tris buffer in 0·45% sodium chloride as the outer phase. Unlabelled progesterone and [1,2-^{3}H] progesterone (S.A. 33 Ci/nmol, New England Nuclear, Boston, Mass., U.S.A.) were added to the outer phase. The dialysis was performed at + 7°C for 38 h, as will be described in the following three experimental series.

In the *first* set of experiments the binding material in the inner phase was the 1% human plasma solution. In all experiments 0·05 μCi of [^{3}H]progesterone was added to all dialysing vessels. The additional amounts of unlabelled progesterone in the outer phase were 0, 10 and 30 ng. Dialysis was allowed to continue for 19 h, after which samples were taken from the inner and outer phases to determine the protein-bound progesterone. Then the purified lecithin solution was added to the outer phase to reach the following concentrations of lecithin in the outer phase: 0, 10, 100 and 1000 mg/100 ml. After the addition of lecithin, dialysis was continued for another 19 h. All experiments were run in duplicate each time.

When the dialysis was discontinued, the inner and outer phases were analysed to determine their concentrations of labelled steroid. Radioactivity was determined in a Wallac Decem-NTL314 liquid scintillation spectrometer (Wallac Oy, Turku, Finland).

In the *second* set of experiments 1% human plasma solution was used in the inner phase, and the outer phase contained 0, 100 or 500 mg/100 ml of lecithin, in addition to the same amounts of labelled and unlabelled progesterone as in the

first experiments, at the beginning of the dialysis. The dialysis was performed for 19 h at +7°C, after which samples were taken from the inner and outer phases and their radioactivities determined. The dialysis was continued for a further 19 h, after which the radioactivity of the inner and outer phases were again determined.

In the *third* set of experiments 1 mg/ml diluted myometrial homogenate from the rats was used as the protein source in the inner phase. The experimental design was otherwise the same as in the first set of experiments, except that the lecithin concentrations were 0, 100 or 500 mg/100 ml.

RESULTS

The radioactivity in progesterone and the total extractable radioactivity in the whole myometrium are given as d.p.m. per 3 g wet weight in Table 1. Table 2 shows the subcellular distribution of progesterone and its free metabolites in the pregnant rat myometrium expressed as d.p.m. per mg of nitrogen in the different experimental groups.

It can be seen that the average total radioactivity in the myometrium is higher in every experimental group of animals than in the control group. This is more

Table 1. Progesterone counts and total radioactivity in pregnant rat myometrial homogenate. Results are expressed as d.p.m. per 3 g wet weight ± S.D. The rats treated with oestradiol received an injection of 2 mg of oestradiol 24 h before the experiments. The amount of intravenous fat infusion was 2 ml of Infonutrol

	Control	Treated with oestradiol	Fat emulsion given before progesterone	Fat emulsion given after progesterone
Progesterone	23,484 ± 3650	37,116 ± 5330	17,030 ± 2778	27,564 ± 4110
Total radioactivity	64,445 ± 8115	101,333 ± 17190	83,158 ± 13250	118,797 ± 15300

Table 2. The distribution of progesterone counts and total radioactivity in different subcellular fractions of pregnant rat myometrium after 1 h of labelled progesterone infusion. The animals treated with oestradiol received an injection of 2 mg of oestradiol intramuscularly 24 h before the experiment. The amount of intravenous fat infusion was 2 ml of Infonutrol. The results are expressed as d.p.m. per mg of nitrogen ± S.D.

	Control		Treated with oestradiol		Fat emulsion given before progesterone		Fat emulsion given after progesterone	
Fraction	progesterone	total radioactivity	progesterone	total radioactivity	progesterone	total radioactivity	progesterone	total radioactivity
Nuclear-myofibrillar	200 ± 24	530 ± 63	267 ± 22	670 ± 69	140 ± 40	650 ± 74	250 ± 35	1120 ± 110
Mitochondrial	110 ± 20	310 ± 34	166 ± 25	450 ± 58	178 ± 20	308 ± 43	94 ± 40	470 ± 89
Microsomes (20,000 *g*)	142 ± 18	330 ± 44	176 ± 32	530 ± 65	106 ± 17	540 ± 69	132 ± 50	640 ± 104
Microsomes (105,000 *g*)	82 ± 19	225 ± 32	190 ± 29	455 ± 55	102 ± 16	272 ± 42	205 ± 45	910 ± 87
Supernatant	74 ± 13	232 ± 30	100 ± 15	345 ± 57	53 ± 11	345 ± 45	67 ± 24	257 ± 90

marked in the animals primed with oestradiol and given Infonutrol after the progesterone than in those which were treated with Infonutrol before progesterone. In the last-mentioned animals less progesterone was retained by the myometrium than in the control animals, whereas with the oestradiol-primed animals more progesterone was retained in the myometrium than in the control rats.

The subcellular distribution of progesterone also seems to be changed in the animals treated with estradiol or Infonutrol. In every subcellular fraction of myometrium in the rats primed with oestradiol there was a higher progesterone concentration per mg of nitrogen than in the control animals. The increase was highest in the microsomes sedimented at 105,000 *g* and the relative increase as compared with the controls was smallest in the microsomes sedimented at 20,000 *g*. However, if the radioactivity as progesterone is compared with the total extractable radioactivity, the ratio is very similar to that found in the subcellular fractions in the control groups (Table 3).

When the lipid infusion was given before progesterone administration, the progesterone per mg of nitrogen was increased in the mitochondrial fraction and in the microsomal fraction sedimented at 105,000 *g*, whereas in the other fractions it was decreased. The ratio between the radioactivity as progesterone and the total extractable radioactivity was also changed in every other fraction except the microsomes obtained at 105,000 *g* (Table 3).

Table 3. The ratios between progesterone counts and total radioactivity in the subcellular fractions of rat myometrium calculated from the results presented in Table 2

	Control	Treated with oestradiol	Fat emulsion given before progesterone	Fat emulsion given after progesterone
Nuclear-myofibrillar	0·38	0·40	0·22	0·22
Mitochondrial	0·35	0·37	0·58	0·20
Microsomes (20,000 *g*)	0·43	0·33	0·20	0·21
Microsomes (105,000 *g*)	0·36	0·42	0·38	0·23
Supernatant	0·32	0·29	0·15	0·26
Total myometrium	0·36	0·37	0·20	0·23

When the lipid infusion was given after the progesterone treatment to study its effect on established progesterone distribution in the myometrium, it was observed that the radioactive progesterone per mg of nitrogen was higher in the nuclear-myofibrillar fraction and in the microsomal fraction obtained at 105,000 *g* than in the controls, whereas in the other fractions it was slightly decreased. There were more metabolites of progesterone in every fraction than in the control series (Table 2).

The results of the equilibrium dialysis experiments are given in Tables 4–6. The effect of the lecithin fraction on the established binding of progesterone by the diluted human plasma solution can be seen from Table 4. There was no effect when the concentration of lecithin in the outer phase was below 100 mg per 100 ml. With a lecithin concentration of 100 mg/100 ml there was a slight

Table 4. Equilibrium dialysis. Inner phase: 1% female plasma. Outer phase: 0·05 μCi [^{3}H]progesterone and 0, 10, 30 ng of cold progesterone. The time of dialysis was 19 h, after which lecithin was added to make a concentration of 0, 10, 100 or 1000 mg/100 ml. Dialysis was then continued for another 19 h. Results are expressed as the ratio of c.p.m. in the outer phase to c.p.m. in the inner phase

Number of experiments	Cold progesterone in outer phase ng	Lecithin added to outer phase mg/100 ml	c.p.m. outer phase / c.p.m. inner phase before addition of lecithin	after addition of lecithin
1	0	0	0·08	0·06
1	10	0	0·13	0·11
1	30	0	0·24	0·20
2	0	10	0·08	0·06
2	10	10	0·13	0·13
2	30	10	0·22	0·21
2	0	100	0·08	0·11
2	10	100	0·13	0·17
2	30	100	0·21	0·27
2	0	1000	0·12	5·69
3	10	1000	0·17	5·37
3	30	1000	0·32	5·68

Table 5. Equilibrium dialysis. Inner phase: 1% female plasma. Outer phase: 0·05 μCi [^{3}H]progesterone; 0, 10, 30 ng of cold progesterone and lecithin in concentrations of 0, 100 and 500 mg/100 ml. The time of dialysis was 38 h. Samples were taken after 19 h and after 38 h of dialysis. Results are expressed as the ratio of c.p.m. in the outer phase to c.p.m. in the inner phase

Number of experiments	Cold progesterone in outer phase ng	Lecithin in outer phase mg/100 ml	c.p.m. outer phase / c.p.m. inner phase after 19 h of dialysis	after 38 h of dialysis
1	0	0	0·08	0·06
1	10	0	0·18	0·19
1	30	0	0·27	0·24
2	0	100	0·79	0·82
2	10	100	1·40	1·40
2	30	100	0·95	1·00
2	0	500	3·95	3·40
2	10	500	3·75	3·75
2	30	500	3·89	3·47

release of radioactive progesterone from the inner phase. However, the concentration of 1000 mg per 100 ml of lecithin in the outer phase resulted in 50-fold enrichment of the radioactivity in the outer phase. The addition of lecithin to the outer phase at the start of dialysis had a marked effect when compared with the results of experiments in which lecithin was added only after binding of progesterone had already been established. However, the enrichment of the radioactivity

Table 6. Equilibrium dialysis. Inner phase: pregnant rat myometrial homogenate; protein concentration 1 mg/ml. Outer phase: 0·05 μCi [^{3}H] progesterone and 0, 10, 30 ng cold progesterone. The time of dialysis was 19 h, after which lecithin was added to make concentrations of 0, 100 and 500 mg/100 ml. Dialysis was then continued for another 19 h. Results are expressed as the ratio of c.p.m. in the outer phase to the c.p.m. in the inner phase

Number of experiments	Cold progesterone in outer phase ng	Lecithin added to outer phase mg/100 ml	c.p.m. outer phase / c.p.m. inner phase before addition of lecithin	c.p.m. outer phase / c.p.m. inner phase after addition of lecithin
1	0	0	0·43	0·47
1	10	0	0·47	0·52
1	30	0	0·52	0·57
2	0	100	0·42	1·46
2	10	100	0·46	1·43
2	30	100	0·54	1·47
2	0	500	0·43	4·10
2	10	500	0·45	4·16
2	30	500	0·53	4·42

in the outer phase with the lecithin concentration of 500 mg per 100 ml (Table 5) was not so great as with 1000 mg/100 ml in the first experimental series (Table 4).

The diluted myometrial homogenate from the pregnant rat was a weaker binder of progesterone than 1% plasma solution, as judged on the basis of experiments without lecithin. The effect of addition of lecithin to the outer phase was also more prominent than when plasma was used as the protein source in the inner phase. Already the concentration of 100 mg of lecithin/100 ml in the outer phase resulted in a significant transfer of the bound progesterone to the outer phase.

DISCUSSION

The results reported here demonstrate that an intravenous infusion of fat emulsion to pregnant rats is able to alter the distribution of radioactive progesterone in the subcellular fractions of the myometrium. In the control rats the subcellular distribution of labelled progesterone was similar to that observed earlier [5]. It was also found that the amount of labelled progesterone metabolites in the myometrium increases after the infusion, indicating that progesterone metabolism is altered as a result of the phospholipid treatment. It has been reported earlier [1, 2] that lecithin is the component of the fat emulsion responsible for uterine stimulation.

The equilibrium dialysis experiments in the present study demonstrated that it is the lecithin component of the fat emulsion that releases progesterone from the specific binding sites in human plasma proteins and in rat myometrial homogenate, and concentrates it in the outer phase. The mechanism by which lecithin exerts its effects in the dialysis experiments [10] is obscure. It could be a result of micelle formation in the lecithin-containing solution, because at a certain lecithin concentration progesterone is completely trapped in the outer phase. It has already been observed that the intravenous fat emulsion used in this study is able to precipitate

premature labour in *rabbits*[1, 2], to increase uterine activity in women during early pregnancy[3] and to induce labour at term[4]. It is tempting to correlate the uterine-stimulating action of the phospholipid fraction with the alteration of myometrial progesterone distribution by this fraction, because the subcellular distribution of progesterone in the myometrium is also altered by delivery[6]. Thus the changing pattern of progesterone distribution in the myometrium may be connected with the physiological actions of this steroid on this target tissue of progesterone.

It should be realized that in the organism infusion of phospholipids affects other physiological processes besides those connected with steroid metabolism, and these other effects could contribute to the uterine-stimulating action of this treatment. However, it is interesting to note that lecithin has yet another action on steroid metabolism, because it has been shown[11] that lecithin is a potent inhibitor of the cholesterol side-chain cleavage enzyme system in mitochondria of human placenta, suggesting that lecithin might inhibit progesterone biosynthesis. In our earlier investigation[3], however, we were unable to demonstrate any decrease of plasma progesterone concentration in early human pregnancy during phospholipid infusion.

The higher uptake of progesterone by myometrium found in this study after priming is in accordance with the findings that oestrogen pretreatment increases the progesterone uptake in the uterus but not in other organs[12] and the total progesterone binding in the uterus[13] of guinea-pigs.

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DISCUSSION

Tuohimaa: I have a minor question to Dr. Wichmann. Into what artery have you injected the fat emulsion, and why have you injected it intraarterially?

Wichmann: If we injected it into the aorta, the concentration of the radioactivity in myometrium was much greater than if we injected it intravenously. All the

radioactivity we recovered in these experiments was about 0·25%, and especially if we tried to inject it above the branch of the renal artery. The arterial flow goes through both the ovarian and the uterine arteries to the myometrium, and the recoveries were much higher in that way compared with injections into a jugular vein or into the tail vein. The injection of lipids was done in the same way as the labelled steroids.

Tuohimaa: If you are injecting into the aorta, is it not possible that the lipid material causes some lipid embolism in the uterine arteries, and that this might explain the lower uptake of the progesterone?

Wichmann: The retention of progesterone was lower, compared with the uptake of the metabolites, so it's difficult to say whether this dose (2 ml) was too much for a rat weighing about 250 g, but the overall activity found was higher in those rats treated with Infonutrol. It was only the ratio between progesterone and the total radioactivity which was changed.

Siiteri: Dr. Haukkamaa, I think this is a very interesting approach to an important problem. In view of our experiences with estrogen-binding proteins in the human endometrium, which indicate that they are rather unstable, I wonder if you've done any short-term experiments to investigate this particular problem which may be relevant to the variability in the results you have obtained with the hyperplastic and the carcinomatous endometrium.

Haukkamaa: I don't know if I've quite understood your question; but I've only studied progesterone, and have no data about estrogen binding. We have not done any short-term experiments.

Jensen: Many of your exposures were for quite a long time. Does your receptor decompose at all during this long exposure, or is it stable?

Haukkamaa: It seems to be stable: if we have this 36 h incubation, we get a few per cent lowering in binding percentage during this time. We have the controls which showed this.

Jensen: Do you have to incubate so long to get complete equilibration?

Haukkamaa: We haven't tried what would be the limit of the incubation time, but if we took some hours, say 10 h, then it would have been difficult for the working routine. We found that these proteins don't degrade, so we think that it doesn't matter if we have a few hours more.

Rosner: Just a small theoretical point in your data analysis. It's not appropriate to talk about binding inside the bag in the albumin experiment. Since you have bound species on both sides of the membrane it is not possible to calculate the percentage of bound steroid in the bag.

Haukkamaa: We could have expressed the results as ratios of c.p.m. or we could have plotted them in Scatchard plots, but I didn't want the last mentioned because we had too few samples, therefore we would have got too few points. So I would like to get more material before calculating these plots. Actually the intention of this investigation was to work with a simple system to see what's the tendency, and then to go on.

Jensen: Dr. Wichmann, if I understood you correctly, your observations were confined to the myometrium in these pregnant rats. What happens in the endometrium? There must be considerable binding there as well?

Wichmann: No, we haven't looked at the endometrium; we scraped it away every time. This investigation is confined only to the myometrial tissue. The metabolism in the endometrium is perhaps 50–100 fold higher than in myometrium.