Immunoblot analysis of cholesterol side-chain cleavage cytochrome P-450 and adrenodoxin in corpora lutea of cyclic and late-pregnant sheep*

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Summary. The specific contents of cytochrome P-450_{sc} and adrenodoxin in corpora lutea of late pregnant sheep were, respectively, 1/5 and 1/8 that of corpora lutea of the oestrous cycle, suggesting lower steroidogenic enzyme capacity in the former. The contents of Complex V proteins were also lower in the corpora lutea of late pregnancy. It was observed in the immunoblots of both Complex V and cytochrome P-450_{sc} that immunoreactive bands of molecular weights lower than the native proteins were present in the samples from corpora lutea of late pregnancy, indicative of degradation of the native enzymes. It is concluded that corpora lutea of sheep during late pregnancy have a much lower enzyme capacity for steroidogenesis than do those of the oestrous cycle (mid-luteal phase) due to a reduction in the content of cytochrome P-450_{sc} and adrenodoxin. The reduction in the levels of steroidogenic enzyme proteins appears to be unspecific and probably reflects an overall demise in mitochondrial functions.

Keywords: cholesterol side-chain cleavage cytochrome P-450; adrenodoxin; corpus luteum; pregnancy; sheep

Introduction

It is well established that elevated plasma concentrations of progesterone are necessary for maintenance of pregnancy in sheep. Progesterone is secreted by the corpus luteum which, if removed during the first 50 days of gestation (total gestational period 145–150 days) will cause abortion of the fetus. If removal is after 50 days of gestation the pregnancy will generally continue (Casida & Warwick, 1945) because the placenta is then able to produce progesterone in amounts sufficient for the maintenance of the pregnancy (Linzell & Heap, 1968; Ricketts & Flint, 1980). Although the corpus luteum is not necessary after Day 50 of gestation it persists throughout pregnancy. Relatively little is known about luteal steroidogenic enzyme capacity in late pregnancy but it would appear that the corpus luteum contributes much less to maternal plasma progesterone concentrations than does the placenta (Bedford *et al.*, 1972), since the concentrations of progesterone in the ovarian venous plasma and the ovarian progesterone secretion rates are reduced drastically in sheep in late gestation (Moore *et al.*, 1972; Harrison & Heap, 1978; Flint *et al.*, 1983). In the present study we have used Western immunoblot techniques to measure the amounts of cholesterol side-chain cleavage cytochrome P-450 (cytochrome P-450_{sc}), the mitochondrial rate-limiting enzyme in the conversion of cholesterol to progesterone, and its electron donor, adrenodoxin, in corpora lutea of late pregnant sheep.

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Materials and Methods

Tissues. Ovaries were collected from multiparous mixed Western breeds of sheep under barbiturate (pentobarbitone) anaesthesia. Corpora lutea, which appeared to be fully formed and functional, based upon their size and colour, from mid-oestrous cycle non-pregnant ewes ($N=5$) and corpora lutea from ewes ($N=5$) late in pregnancy (133 ± 4 days; mean $\pm$ s.e.m.) were obtained. All animals had a single unilateral corpus luteum. Gestation length was determined from the day of mating (Day 0) and confirmed by the crown-rump length of the fetus. Corpora lutea were quickly frozen in liquid nitrogen stored frozen (-70°C) until the day of analysis and then thoroughly homogenized with a glass homogenizer in phosphate-buffered (0.1 M, pH 7.4) saline containing 5 mM-ethyleneglycol-bis- β -aminoethylether *N,N'*-tetraacetic acid (EGTA), 10 mM-dithiothreitol, 0.1 M-leupeptin, 0.2 mM-phenylmethylsulphonylfluoride (PMSF) and 1% sodium dodecyl sulphate (SDS) at 10 ml/g tissue. The homogenates were sonicated (pulsed, 1 min) and then centrifuged (800 *g*) to remove any tissue debris.

The protein concentrations of the supernatants were determined by the method of Lowry *et al.* (1951) and the yield of protein from corpora lutea of both cyclic and late-pregnant ewes was similar ($115.4 \pm$ s.e.m. 19 versus 140.2 ± 5.7 $\mu\text{g}/\text{mg}$ wet weight). The specific contents of cytochrome P-450_{sec}, adrenodoxin and Complex V (Stigall *et al.*, 1977) were determined by Western immunoblot analyses.

Immunoblotting. Tissue homogenates (50 μg protein), molecular weight standards (Bio-Rad Laboratories, Richmond, CA, U.S.A.), purified standards and samples of corpora lutea from cattle and rats that were useful for identification of specific proteins were each mixed with an equal volume of Tris buffer (pH 6.8, 50 mM) containing 2% SDS, 10% glycerol, 0.4 M-mercaptoethanol, 2 mM-ethylenediamine-tetra-acetic acid (EDTA) and 0.05% bromophenol blue and boiled for 10 min. Proteins were then separated according to their molecular weight by electrophoresis on polyacrylamide gels (10% for cytochrome P-450_{sec} and Complex V and 15% for adrenodoxin) containing SDS. The separated proteins were then electroblotted onto nitrocellulose paper for 12–18 h at 4°C in 20% methanol, 20 mM-Tris base and 150 mM-glycine at 175 mA. Nitrocellulose blots were incubated with 5% bovine serum albumin in Tris-buffered (pH 7.4, 10 mM) saline containing 0.2% Nonidet P-40 (37°C , 45 min), and further incubated in fresh buffer containing primary rabbit IgG (room temperature, 2 h). For each blot, 70 μg primary IgG in 35 ml buffer were used except for Complex V for which 4–2 μl whole serum were used. The blots were then rinsed (3×10 min) in Tris-buffered (pH 7.4, 10 mM) saline containing 0.2% Nonidet P-40, 0.25% sodium deoxycholate and 0.1% SDS and then further incubated with ^{125}I -labelled goat anti-rabbit IgG in buffer containing bovine serum albumin as above (room temperature, 45 min). The secondary goat anti-rabbit IgG was labelled by the lactoperoxidase method (Thorell & Johansson, 1971) and used at 35×10^6 c.p.m. per 35 ml buffer. Because more bands of protein were observed in blots of protein from corpora lutea of pregnancy, some blots were repeated with another batch of secondary IgG, yielding the same results and showing that these bands were not likely to be due to artefactual non-specific binding of the secondary IgG. Immunoblots were stained with amido black (0.5 mg/ml in 45% methanol and 10% acetic acid solution) to locate the position of the molecular weight markers. Autoradiograms of immunoblots were obtained using Kodak AR film with intensifying screens and exposure times of 6–18 h. Autoradiograms were scanned densitometrically and the intensity of the specific bands was used as an estimate of the amount of the specific protein. Earlier experiments using bovine corpora lutea had shown that the intensities of the autoradiographic bands for either cytochrome P-450_{sec} or adrenodoxin were linearly related to the amounts of homogenates loaded on the gels and parallel to dilutions of purified standards. The maximum amount of homogenate tested in these experiments was 50 μg protein and the intensities obtained in the present experiments were not outside the linear range.

Purified protein standards and antibodies. Cytochrome P-450_{sec} (Seybert *et al.*, 1979) and adrenodoxin (Lambeth & Kamin, 1979) were purified from bovine adrenal cortex and kindly donated by Dr J. D. Lambeth, Emory University, Atlanta, GA, U.S.A. Antisera were raised against these proteins and IgG fractions were prepared (DuBois *et al.*, 1981a, b). The antisera have been used previously for immunoisolations of radiolabelled cytochrome P-450_{sec} and adrenodoxin from a number of steroidogenic tissues of various species (Kramer *et al.*, 1982; Funkenstein *et al.*, 1983, 1984; Ohashi *et al.*, 1983; Veldhuis *et al.*, 1986), for immunoisolations from cell-free translations of mRNA (DuBois *et al.*, 1981b), for tissue localization studies (Kramer *et al.*, 1984; Rodgers *et al.*, 1986a, b), for immunoblotting techniques (Rodgers *et al.*, 1986c; Trzeciak *et al.*, 1986) and for isolation of cDNA clones for cytochrome P-450_{sec} (John *et al.*, 1984) and adrenodoxin (Okamura *et al.*, 1985). Complex V proteins, which include the subunits of F_1 -ATPase, were purified by the methods of Stigall *et al.* (1977) and antisera against Complex V proteins were raised in rabbits and kindly donated by Dr G. A. Breen, University of Texas at Dallas, TX, U.S.A.

Results

The Western immunoblots for cytochrome P-450_{sec}, adrenodoxin, and Complex V proteins are shown in Figs 1, 2 and 3, respectively. The order of samples from corpora lutea of mid-oestrous cycle non-pregnant and pregnant sheep is the same on each of the immunoblots. Gestational ages

of the pregnant ewes were 125, 127, 127, 141 and 145 days and are presented from left to right in all figures.

Fig. 1. Autoradiogram of Western immunoblot of proteins (50 µg/lane) from corpora lutea (CL) of 5 non-pregnant ewes and 5 late-pregnant ewes developed using anti-cytochrome P-450_{sec} IgG. Arrow indicates the position of purified bovine cytochrome P-450_{sec}.

Cytochrome P-450_{sec}

The anti-cytochrome P-450_{sec} IgG reacted with proteins of M_r 49 000 (Fig. 1) that co-electrophoresed with purified bovine adrenal cytochrome P-450_{sec} and with cytochrome P-450_{sec} in homogenates of bovine and rat corpora lutea (data not shown), identifying cytochrome P-450_{sec} of ovine corpora lutea in the Western immunoblot. The IgG also reacted with proteins of M_r 45 000–46 000 from corpora lutea of the non-pregnant ewes and the late-pregnant ewes (Fig. 1). Substantial proportions of the tissue proteins were present in the same area as judged by the intensity of amido black staining. This immunoreactive band has been immunisolated from cultured cow luteal cells and pig granulosa cells with IgG against other proteins (unpublished observations) and is not considered to be related to cytochrome P-450_{sec}. The levels of cytochrome P-450_{sec} were similar in

Fig. 2. Autoradiogram of Western immunoblot of proteins (50 μ g/lane), from corpora lutea (CL) of 5 non-pregnant ewes and 5 late-pregnant ewes, developed using anti-adrenodoxin IgG. Arrow indicates the position of purified bovine adrenodoxin.

each corpus luteum of cyclic ewes and were some 7-fold greater than those of the corpora lutea of late-pregnant ewes ($332 \pm \text{s.d. } 50.5$ versus 48.6 ± 38.5 arbitrary units). Moreover, the levels in the corpora lutea of pregnant ewes were much more heterogeneous and the IgG reacted with many more proteins of low molecular weight. These low molecular weight immunoreactive proteins of pregnant ewes may have been identified by the IgG merely as non-specific interactions or more likely, as they have not been observed before in any other steroidogenic tissues, as specific interactions with proteolytic products of cytochrome P-450_{sec}.

Adrenodoxin

The anti-adrenodoxin IgG reacted with a band of M_r approximately 12 000 (Fig. 2). This band was identified as adrenodoxin since it also co-electrophoresed with the purified bovine adrenal adrenodoxin standard, which was of a slightly faster electrophoretic mobility than adrenodoxin in homogenates of bovine corpora lutea (Rodgers *et al.*, 1986c). The levels of adrenodoxin were relatively similar among the corpora lutea of cyclic ewes and were more than 8-fold higher than those of the corpora lutea of late-pregnant ewes (213.4 ± 54.6 versus 25.2 ± 4.8 arbitrary units).

Complex V proteins

Complex V consists of a group of proteins from the inner mitochondrial membrane (Stigall *et al.*, 1977) and the antiserum used was capable of recognizing some of them (Fig. 3). Three proteins were readily identified on Western blot analysis of corpora lutea of the oestrous cycle as bands corresponding to M_r 50 000, 47 000 and 34 000. The amounts of the proteins of M_r 50 000 and 47 000 in corpora lutea of late-pregnant ewes were reduced, but that of M_r 34 000 was reduced in only 2 of the 5 corpora lutea of late-pregnancy. In the corpora lutea of late-pregnant ewes only

Fig. 3. Autoradiogram of Western immunoblot of proteins (50 µg/lane) from corpora lutea (CL) of 5 non-pregnant ewes and 5 late-pregnant ewes developed using an antiserum against Complex V proteins.

the antiserum also recognized a number of other proteins at lower molecular weights, consistent with the notion that these proteins were the degradation products of the higher molecular weight complex V proteins.

Test for proteolysis artefact

Since it is suggested from the immunoblot analyses that the inner-mitochondrial proteins of corpora lutea of late-pregnant ewes had been degraded it was necessary to determine whether this was an artefact due to the release of proteolytic enzymes upon homogenization and whose activity was not inhibited by the protease inhibitors in the buffer. Samples of corpora lutea of cyclic and late-pregnant ewes were paired and an aliquant from one sample of the pair (equivalent to 25 µg protein) was taken and then combined with the other of the pair and incubated at 37°C for 90 min. The samples were then subjected to immunoblotting analyses for cytochrome P-450_{scc}, adrenodoxin and Complex V proteins and compared with the results of the parent samples. There was no evidence that the enzymes of the cyclic corpora lutea were degraded by incubation with homogenates of late-pregnancy corpora lutea, suggesting that proteolytic enzymes were not active in these homogenates.

Discussion

In the present study it was found that the specific tissue contents of the rate-limiting enzyme in the conversion of cholesterol to progesterone, cytochrome P-450_{sc}, and its electron donor, adrenodoxin were substantially lower in sheep corpora lutea during late pregnancy than during the oestrous cycle of non-pregnant ewes. It is therefore suggested that the sheep corpus luteum has a much lower enzyme capacity for steroidogenesis in late pregnancy than during the oestrous cycle. Moreover, the reduction in the tissue content of cytochrome P-450_{sc} and adrenodoxin did not appear to be specific for these proteins because the contents of other 'house-keeping' inner-mitochondrial membrane proteins were also reduced.

In the corpora lutea of the oestrous cycle the tissue levels of cytochrome P-450_{sc} and adrenodoxin were relatively homogeneous but in the corpora lutea of late-pregnant ewes the levels of cytochrome P-450_{sc} were very heterogeneous and there was no consistent pattern with advancing gestation. In the immunoblots of cytochrome P-450_{sc} and Complex V proteins it was observed that the antibodies reacted with a number of different low molecular weight proteins in samples of corpora lutea of late-pregnant ewes but not in those of corpora lutea of the non-pregnant ewes. It is possible that these proteins were breakdown products of the native enzymes. If they were then it is unlikely that they were formed by the release of proteolytic enzymes upon homogenization since the buffer used for homogenization contained proteolytic enzyme inhibitors (EGTA, dithiothreitol, leupeptin and PMSF) and no proteolytic activity could be demonstrated in the homogenates. It is therefore likely that these degradation products existed *in vivo* and account in part for the low levels of native enzymes in corpora lutea of late-pregnant ewes. Of course the low levels of the enzymes as a proportion of total cell proteins could be explained by a lower volume density of mitochondria. However, it is not known whether there is a reduction in the volume density of mitochondria in late pregnancy.

Mitochondria of luteal cells from the mid-luteal phase of the oestrous cycle in the sheep appear as typical 'steroidogenic mitochondria' with tubular/vesicular cristae and small electron-dense matrix granules (Bjersing *et al.*, 1970a; Nett *et al.*, 1976; O'Shea *et al.*, 1979) of the type often associated with calcium storage (Hopkins, 1978). In corpora lutea of late-pregnant ewes a significant proportion of mitochondria have in their matrix large rounded granules of electron-density greater than that of the lipid droplets and of such size that they occasionally exceed 1 µm in diameter and almost fill the entire area bounded by the inner membrane (Bjersing *et al.*, 1970b; O'Shea *et al.*, 1979). The cristae are often less well developed and are more lamellar in these mitochondria (O'Shea *et al.*, 1979). From the present study, it is suggested that these mitochondria are functionally impaired in terms of the content of inner-mitochondrial membrane enzymes, particularly the cholesterol side-chain cleavage and Complex V proteins.

In support of this, concentrations of progesterone in the ovarian venous plasma of sheep in late gestation have been reported to be nearly one tenth that observed in early or mid-gestation (Moore *et al.*, 1972; Flint *et al.*, 1983). Furthermore, Harrison & Heap (1978) reported that the ovarian secretion rate of progesterone *in vivo* was reduced between Days 122 and 145 of gestation when compared to early pregnant animals, or non-pregnant sheep in the luteal phase. This demise in luteal function late in gestation is also associated with a dramatic decrease in the weight and total progesterone content of the corpus luteum of pregnancy (Moore *et al.*, 1972). Bjersing *et al.* (1970b) also found very low plasma concentrations of progesterone in hysterectomized ewes with corpora lutea 137–146 days old. Furthermore, these corpora lutea had mitochondria with the same type of matrix granules as observed in late pregnancy (Bjersing *et al.*, 1970a). In the cow, corpora lutea of late pregnancy have similar mitochondrial matrix granules (Sorenson & Singh, 1973; Singh, 1975) which increase in occurrence with length of gestation (Fields *et al.*, 1985). Luteal cells from corpora lutea of pregnant cows produce significantly less progesterone than do those of non-pregnant animals (Weber *et al.*, 1984), adding further support to the suggestion that these mitochondria have a reduced ability to metabolize cholesterol into progesterone due to the lack of active cholesterol side-chain cleavage enzyme. It still remains a matter of speculation as to the composition of the

granules. If they are composed of lipid or degraded proteins or both then their formation in mitochondria would probably be as a consequence of, rather than a cause of, the reduction in the luteal content of cytochrome P-450_{sc} and adrenodoxin.

In conclusion, the corpus luteum of the late pregnant sheep has a much lower enzyme capacity for steroidogenesis due to a reduction in the tissue contents of cytochrome P-450_{sc} and adrenodoxin than does that of the cyclic ewe. However, this reduction may not be specific to the steroidogenic proteins because there is a reduction in the contents of other inner-mitochondrial membrane enzymes as well as morphological changes, reflecting an overall demise in mitochondrial function in late gestation.

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