LUTEOLYSIS IN NORMAL AND PROSTAGLANDIN $F_{2\alpha}$ -TREATED PSEUDOPREGNANT RABBITS

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Summary. Prostaglandin $F_{2\alpha}$ caused both a morphological regression of the corpus luteum and a decrease in peripheral progesterone levels within 24 hr after administration to rabbits on Day 8 of pseudopregnancy. The degenerating luteal cells contained autophagic vacuoles and an abundance of whorled smooth endoplasmic reticulum which transformed into myelin figures. The normal regression observed at the end of pseudopregnancy (Day 16, 18 and 20) was characterized by a decrease in whorled smooth endoplasmic reticulum and by an increase in the number of lipid droplets. The appearance of these droplets changed from moderately dense to lucent during this period. Autophagic vacuoles and myelin figures were not prevalent, suggesting that the lytic process may differ between normal and prostaglandin $F_{2\alpha}$ -induced luteolysis.

INTRODUCTION

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been shown to cause functional luteolysis in several species (see Pharriss, Tillson & Erickson, 1972). A decrease in luteal activity has also been shown to be accompanied by distinct changes in CL morphology in the pregnant rabbit (Koering & Kirton, 1973). The CL was shown to be most responsive to $PGF_{2\alpha}$ at mid-pregnancy when the luteal cells were filled with whorled smooth endoplasmic reticulum (Koering & Kirton, 1973).

The CL in the rabbit is maintained by oestrogen (Spies & Quadri, 1967; Keyes & Nalbandov, 1967) and therefore any factor interfering with the effect of oestrogen will result in CL regression. More recently, a cytoplasmic oestrogen receptor has been identified in the CL of pseudopregnant rabbits and has been found to be at highest concentration at mid-pseudopregnancy (Lee, Keyes & Jacobson, 1971). If the rabbit is treated with $PGF_{2\alpha}$ at this time, the concentration of receptor decreases (Jacobson, Bullock & Keyes, 1972).

This study was undertaken to determine the effect of $PGF_{2\alpha}$ on the CL at mid-pseudopregnancy which may possibly be correlated with the decrease in receptor concentration, and to identify the changes occurring during normal CL regression.

MATERIALS AND METHODS

Fifteen pseudopregnant New Zealand rabbits were randomly assigned to one of four groups (Day 9, 16, 18 and 20 of pseudopregnancy). The animals in the Day-9 group (mid-pseudopregnancy) were in pairs with one receiving 400 μg $\text{PGF}_{2\alpha}$ dissolved in gelatin/kg subcutaneously 24 hr before autopsy while the other was given an equivalent amount of vehicle (Jacobson *et al.*, 1972). The remaining rabbits were killed on Day 16, 18 or 20. Each animal was anaesthetized with Nembutal (Abbott) and then perfused through the descending aorta with 400 ml fixative. The perfusion fluid contained a combination of paraformaldehyde (4%), glutaraldehyde (1%), glucose (5.4%) and calcium chloride (1 mM) in 0.1 M-cacodylate buffer at pH 7.4. Both ovaries were then removed and portions of the CL were prepared for electron microscopy by immersion in formaldehyde-glutaraldehyde-2,4,6-trinitrophenol (Ito & Karnovsky, 1968) in 0.1 M-cacodylate buffer with 2 mM-calcium chloride and minced into 1 to 2 mm³ pieces. The remainder of the ovarian tissue was placed in

EXPLANATION OF PLATES 1 TO 4

PLATE 1

FIG. 1. A 1- μm thick Epon-embedded section of a CL from a control Day-9 pseudopregnant rabbit. The luteal cells are large and surrounded by endothelial cells (E). The dark structures (circle) in the cytoplasm are lipid droplets.

FIG. 2. A portion of a luteal cell from a control Day-9 pseudopregnant rabbit. Lipid droplets (L) are located between two whorled smooth endoplasmic reticulum structures. The membranes of the whorl are seen in cross-section showing them to be composed of tubules (arrows) as well as layers.

FIG. 3. A portion of a luteal cell from a control Day-9 pseudopregnant rabbit. The periphery of the cell is bordered by a blood vessel (V). Many of the lipid droplets are surrounded by whorled smooth endoplasmic reticulum (W). The mitochondria are elongated and often flattened centrally (F).

PLATE 2

FIG. 4. A 1- μm thick Epon-embedded section of a CL from a $\text{PGF}_{2\alpha}$ -treated rabbit at Day 9 of pseudopregnancy. The luteal cells are irregular in shape and large dark structures can be identified (arrows).

FIG. 5. Portion of a luteal cell from a $\text{PGF}_{2\alpha}$ -treated rabbit at Day 9 of pseudopregnancy. The cytoplasm appears normal, although three autophagic vacuoles are present. One vacuole contains two lipid droplets (L) and has a dense matrix. Another has four lucent vacuoles (V) and many mitochondria, while another is composed of loose membranes, small vesicles and spherical mitochondria (M).

PLATE 3

FIG. 6. Portion of a luteal cell from a $\text{PGF}_{2\alpha}$ -treated Day-9 pseudopregnant rabbit. The autophagic vacuole is separated from the surrounding luteal cell cytoplasm by a double membrane (arrows). A myelin figure (M) can be seen with part of its membrane in the normal cytoplasm and part in the vacuole. The content of the lipid droplets in the vacuole is flocculent (F) while the droplets in the normal cytoplasm are of medium density (L).

FIG. 7. Portion of a luteal cell from a $\text{PGF}_{2\alpha}$ -treated Day-9 pseudopregnant rabbit showing a myelin figure. This structure is composed of concentric whorls of smooth membranes and surrounds distorted lipid droplets (L) and some rough endoplasmic reticulum (R). Some normal whorled smooth endoplasmic reticulum (S) is seen on the periphery and appears to be contributing to the tightly packed membranes.

PLATE 4

FIG. 8. Portion of a CL from a $\text{PGF}_{2\alpha}$ -treated Day-9 pseudopregnant rabbit. Scattered between vessels (V) are portions of luteal cells which contain whorled smooth endoplasmic reticulum (W) and apparent autophagic vacuoles. These structures are surrounded by a double membrane (arrows) and are composed of lipid droplets (L), spherical mitochondria and myelin figures (F).

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Bouin's fixative and processed for light microscopy to determine the overall appearance of the ovarian compartments. The minced samples were fixed for 4 hr, washed in 0.1 M-cacodylate buffer (pH 7.4) with 2 mM-calcium chloride and post-fixed in buffered 1% osmium tetroxide for 1½ hr. The tissues were dehydrated in ethanol-propylene oxide and embedded in Epon 812. Thick sections (1 µm) were cut and stained with toluidine blue (Trump, Smuckler & Benditt, 1961) to verify the type of tissue. Thin sections were cut on a Reichert OMU2 microtome, placed on bare or coated grids, stained with lead citrate and examined in a RCA-EMU-3H electron microscope.

Peripheral blood was removed from each animal before treatment and/or just before ovariectomy. The serum was frozen and later assayed for progestins by the competitive protein-binding method as previously described (Sobota & Kirton, 1970). As used in this method, petroleum ether extracts 85% progesterone, 45% 17 α -hydroxypregn-4-ene-3,20-dione (17-hydroxyprogesterone) and 65% 20 α -hydroxypregn-4-ene-3-one (20 α -hydroxyprogesterone). Although the binding globulin used has almost equal affinity for progesterone and 17-hydroxyprogesterone, its affinity for 20 α -hydroxyprogesterone is only about 10% that of the other two steroids.

EXPLANATION OF PLATES 5 TO 8

PLATE 5

FIG. 9. A higher magnification of the area within the frame in Pl. 4, Fig. 8. A double membrane surrounds most of the apparent autophagic vacuoles but occasionally the vacuoles are in direct contact with the sub-endothelial spaces (arrows). The vacuoles are composed of lipid droplets (L) with a flocculent content, spherical mitochondria (M) and other membranous structures.

FIG. 10. A portion of a CL from a $\text{PGF}_{2\alpha}$ -treated Day-9 pseudopregnant rabbit. A neutrophil is located in the sub-endothelial space and lies adjacent to various cells and two vessels (V). The multilobed nucleus is surrounded by cytoplasm composed of various granules (G) and glycogen (g).

PLATE 6

FIG. 11. A 1-µm thick Epon-embedded section from a CL at Day 16 of pseudopregnancy. Endothelial cells (C) surround the blood vessels. The luteal cells are large and their cytoplasm contains lipid droplets in which the content is either extracted (E) or retained.

FIG. 12. A 1-µm thick Epon-embedded section from a CL at Day 18 of pseudopregnancy. Blood vessels (V) are dispersed between luteal cells of various sizes. In some cells, the content is extracted (E) while in others it is retained.

FIG. 13. A 1-µm thick Epon-embedded section from a CL at Day 20 of pseudopregnancy. The boundaries of the luteal cells are not well delineated. All lipid droplets are lucent as their content is extracted and they are of various sizes, some appearing as slits (S).

FIG. 14. A luteal cell from a CL at Day 16 of pseudopregnancy. Blood vessels (V) are located around the cell. The luteal cell has a central nucleus (N), an increased number of lipid droplets (L) of medium density and a decrease in whorled smooth endoplasmic reticulum (compare with Pl. 1, Fig. 3).

PLATE 7

FIG. 15. Portions of several luteal cells from a Day-18 pseudopregnant rabbit. A nucleus (N) is seen in one cell which contains lucent lipid droplets some of which are distorted (D). The adjacent cells have lipid droplets whose content is either of medium density (L) or is flocculent (F). A portion of whorled smooth endoplasmic reticulum (W) is seen in another cell and a myelin figure (M) is also present.

PLATE 8

FIG. 16. Portions of several luteal cells from a Day-20 pseudopregnant rabbit. Parts of vessels (V) are adjacent to the luteal cells. The cell membrane (M) of one cell is folded at its periphery and some of the lucent lipid droplets have enlarged while others become elongated (E). Other membranous structures have compressed into small densely outlined vacuoles (D).

RESULTS

Morphology

Day 9 (Control). At this stage, the CL was composed of large cells, each with a spherical nucleus and many lipid droplets (Pl. 1, Fig. 1). These lipid inclusions were often surrounded (Pl. 1, Fig. 3) by whorls of smooth endoplasmic reticulum (SER). The membranes of these whorls might be fenestrated and in some areas were tubular (Pl. 1, Fig. 2). The remaining parts of each cell had elongated mitochondria, tubular SER, Golgi apparatus and some rough endoplasmic reticulum (Pl. 1, Fig. 3).

Day 9 (Treated). Survey sections of the paraffin wax-embedded whole CL from these animals revealed dark structures distributed throughout. These structures were seen in more detail in the Epon-embedded 1- μ m thick sections and they appeared to be intra- or intercellular. Those present within the luteal cells were autophagic vacuoles (Pl. 2, Fig. 5) and myelin figures (Pl. 3, Fig. 7).

The autophagic vacuoles or cytosegresomes were composed of irregular-shaped mitochondria, vacuoles and whorled membranes (Pl. 2, Fig. 5). Such autophagic structures could be dense and often no limiting membrane was identified (Pl. 2, Fig. 5). In other cells, however, similar autophagic vacuoles were surrounded by a double membrane which segregated them from the rest of the cell which still appeared normal (Pl. 3, Fig. 6). Vacuoles surrounded by a single membrane were less prevalent and were similar to those previously described during pregnancy after treatment with PGF_{2 α} (Koering & Kirton, 1973).

Myelin figures appeared to result from whorled SER and the contents within the whorls became distorted (Pl. 3, Fig. 7). Occasionally, structures resembling dense autophagic vacuoles could be seen communicating with the sub-endothelial space suggesting that they were extracellular. The components of these large structures were similar (Pl. 4, Fig. 8) to those of the smaller intracellular type (Pl. 2, Fig. 5). The large segregated portions could be partly surrounded by more than one luteal cell or even exposed directly to the sub-endothelial space (Pl. 5, Fig. 9). In the same area, neutrophils were recognized by their segmented nucleus and many granules (Pl. 5, Fig. 10).

Day 16. During early regression, distinct signs of luteolysis were observed. The cells were still large but the lipid droplets had increased in number and, in a few cells, the droplets appeared lucent (Pl. 6, Fig. 11). Whorled SER was rarely seen (Pl. 6, Fig. 14) in contrast to the amount in Day 9 CL (Pl. 1, Fig. 3).

Day 18. At this stage, much variability existed in lipid droplet content between adjacent cells (Pl. 7, Fig. 15) although more cells had lucent droplets (Pl. 6, Fig. 12) than were seen on Day 16. Occasionally, small myelin figures were present (Pl. 7, Fig. 15).

Day 20. All lipid droplets were lucent at this time and some remnants were elongated (Pl. 6, Fig. 13). Individual luteal cells could still be identified and the vessels appeared normal (Pl. 8, Fig. 16). The cell membrane tended to fold and the sub-endothelial space was obvious. Many lucent lipid droplets coalesced to form inclusions of various sizes and shapes (Pl. 8, Fig. 16).

Progestins

Progestin levels were (mean $\pm$ S.E.) 12.1 ± 0.9 ng/ml ($N = 3$) on Day 8 and 14.8 ± 2.3 ng/ml ($N = 3$) on Day 9 of pseudopregnancy in untreated rabbits. At the same stages of pseudopregnancy, the progestin levels were 13.7 ± 2.5 ng/ml ($N = 3$) immediately before and 0.7 ± 0.3 ng/ml ($N = 3$) 24 hr after treatment with $\text{PGF}_{2\alpha}$. On Days 16, 18 and 20 of pseudopregnancy, the progestin levels were 1.9 ± 1.6 , 0.0 and 0.4 ± 0.3 ng/ml ($N = 3$ for each group), respectively. (N = the number of rabbits.)

DISCUSSION

$\text{PGF}_{2\alpha}$ -induced luteolysis

The normal CL at mid-pseudopregnancy in this study morphologically and functionally resembled the CL of mid-pregnancy (Koering & Kirton, 1973). At both times, whorled SER was the characteristic feature, although it was less extensive at mid-pseudopregnancy. During mid-pseudopregnancy, the oestrogen receptor is also most concentrated (Lee *et al.*, 1971) and progestin levels are elevated suggesting that a functional relationship may exist between these three parameters. Some of the whorled SER was interrupted by tubules, supporting a previously described configuration (Blanchette, 1966). Similar whorls have been reported in the luteal cells of other species during various functional stages (Bjersing, 1967; Adams & Hertig, 1969; Crisp, Dessouky & Denys, 1970, 1973; Crombie, Burton & Ackland, 1971; Sinha, Seal & Doe, 1971; Enders, 1973; Long, 1973; Koering, Wolf & Meyer, 1973a, b), although tubular SER is always prevalent in all steroid-secreting cells (Christensen & Gillim, 1969) and contains enzymes essential for steroid biosynthesis (Dorfman & Ungar, 1965). The presence of two types of SER suggests that differences may exist in the intracellular mechanisms, as was postulated for the ductus deferens (Hamilton, Jones & Fawcett, 1969). In adrenal steroid-secreting cells, the quantity of tubular SER can be influenced by various stimuli (Shelton & Jones, 1971; Nussdorfer, Mazzocchi & Rebonato, 1971; Nussdorfer, Mazzocchi & Rebuffat, 1973), indicating that the membranes may play a functional rôle.

After the administration of $\text{PGF}_{2\alpha}$ at mid-pseudopregnancy, distinct structural changes occurred in the luteal cells which were similar to those seen after treatment during mid-pregnancy (Koering & Kirton, 1973): small and large autophagic vacuoles (cytosegresomes) were identified. These structures are segregated portions of luteal cell cytoplasm and may be lysosomal in nature (Ericsson, 1969). They are characteristic of many cell types in which remodelling and involution are normal phenomena. Several mechanisms have been postulated to explain their origin (Ericsson, 1969). From observations in the present study, it seems that the double membrane that surrounds some autophagic vacuoles may have originated from preformed membranes of the endoplasmic reticulum. These structures may be released into the sub-endothelial space where they then appear to have a single limiting membrane. Most autophagic vacuoles are dense and contain organelles and inclusions which can be recognized, suggesting minimal degradation. Cytosegresomes can be induced by various mechanical, chemical and hormonal changes (Ericsson, 1969), among

them hypoxia (Ashford & Burdette, 1965) and a decrease in essential hormones as is seen in the involuting mammary gland (Helminen & Ericsson, 1968; Brandes, Anton & Barnard, 1969). It seems possible these conditions are mediated through the circulatory system. This system may also play a major rôle in regulating CL activity. It has been shown that $\text{PGF}_{2\alpha}$ causes a decrease in blood flow in the ovary of the rat, rabbit (Pharriss, 1971) and monkey (Kirton, Gutknecht, Bergstrom, Wyngarden & Forbes, 1973) as well as in the CL of the rabbit (Novy & Cook, 1973). Such a change in vascular dynamics can initiate luteolysis (Goding, Baird, Cumming & McCracken, 1972) and may indicate that $\text{PGF}_{2\alpha}$ can cause CL regression through its effect on the vascular system.

Other characteristic features of CL involution are the appearance of myelin figures and neutrophils. Myelin figures are frequently seen in regressing cells that have an abundance of membranous structures (Trump & Ericsson, 1965), and therefore would be expected to be prevalent where whorled SER occurs. Neutrophils are present in the sub-endothelial spaces, although the actual migration of leucocytes is not observed. It is believed that these cells can migrate through intercellular endothelial spaces following certain inflammatory conditions (Dittrich, 1962; Movat & Fernando, 1963; Grant, 1965; Spicer & Hardin, 1969). Whether the neutrophil invasion is a direct response to $\text{PGF}_{2\alpha}$ is not known at this time, but neutrophils have been described in normal luteolysis in the hamster (Grady & Greenwald, 1968; Leavitt, Basom, Bagwell & Blaha, 1973).

Normal luteolysis

Near the end of pseudopregnancy (Day 16), the majority of luteal cells contain an increased number of lipid droplets, which are of uniform size and medium density, and less whorled SER. The increase in lipid inclusions is characteristic of many involuting CL (Claesson & Hillarp, 1948; Cavazos, Anderson, Belt, Henricks, Kraeling & Melampy, 1969; Leavitt *et al.*, 1973). As luteolysis progresses, these droplets increase in size and gradually become lucent; the content is homogenous for each cell. This phenomenon is also seen in the rhesus monkey (Koering *et al.*, 1973a) and may represent a response to the technical procedures, suggesting a difference in the nature of the lipid content. This variability is supported by previous data which show that total cholesterol increases during regression of rabbit CL (Hilliard, Spies & Sawyer, 1969) and that cholesterol esters increase in the degenerating human CL (Weinhouse & Brewer, 1942). At Day 20, the lucent lipid droplets, which are also referred to as vacuoles, are of various sizes and shapes similar to those occurring in most degenerating luteal cells (Christensen & Gillim, 1969). Myelin figures and large autophagic vacuoles are only occasionally seen, as in the pig (Cavazos *et al.*, 1969) and rat (Long, 1973). This seems to be in contrast to the observations of other authors who describe more of these degenerative structures (Van Lennep & Madden, 1965; Bjersing, 1967; Quatacker, 1971; Crombie *et al.*, 1971; Leavitt *et al.*, 1973) and to the results of luteolysis induced with $\text{PGF}_{2\alpha}$.

The two mechanisms often described for normal luteolysis are autophagy and heterophagy. The former is characterized by primary lysosomes which

can eventually digest parts of the cell itself (DeDuve & Wattiaux, 1966) and the latter is recognized by the presence of macrophages. Primary lysosomes have been observed in sheep and rat luteal cells (Dingle, Hay & Moor, 1968; Long, 1973) but are not characteristic in the rabbit during Days 16 to 20 of pseudopregnancy. Macrophages are believed to be responsible for the ingestion of cellular fragments during the process of involution (Helminen & Ericsson, 1968) and are also thought to participate in luteolysis of rat CL (Dott, 1969). Such an invasion of phagocytic cells was not seen in this study during the stages examined.

Structure-function relationship

At mid-pseudopregnancy in the rabbit, progesterin levels were elevated in the controls but showed a sharp decline in the treated animal 24 hr after administration of PGF_{2α}. These results are in keeping with those of previous studies which indicate high progesterin levels at mid-pseudopregnancy in normal rabbits (Hilliard *et al.*, 1969) but show an obvious decline after the administration of PGF_{2α} (Jacobson *et al.*, 1972). The decline of progesterins at the end of pseudopregnancy is to be expected (Hilliard *et al.*, 1969) but this decrease in progesterin release occurs at a time when very few of the luteal cells (Day 16) display morphological degeneration. It therefore appears that the secretion of progesterins decreases while the luteal cells are filled with lipid droplets.

It seems reasonable to suggest that luteolysis is instigated by variations in the blood flow. This is supported by a recent study which shows that the blood flow to the CL decreases just after mid-pregnancy (Abdul-Karim & Bruce, 1973) when progesterin levels begin to decline (Challis, Davies & Ryan, 1973; Hilliard, Scaramuzzi, Penardi & Sawyer, 1973). Such changes may be initiated locally by PGF_{2α} which has recently been shown to be synthesized within the ovary itself (Wilks, Forbes & Norland, 1973). These data suggest that PGF_{2α} may exert a local effect which ultimately results in a decrease in oestrogen receptor concentrations (Jacobson *et al.*, 1972) as well as in a decline in progesterins associated with morphological changes in the CL.

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