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Ovariectomy-Induced Tubulin Polymerization in Pregnant Rat Mammary Gland[1] (41640)

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Abstract. The pool of polymerized (microtubular) tubulin in the mammary glands of pregnant rats, ovariectomized (OVX) at 18 days, increased approximately threefold between 18 and 24 hr following ovariectomy, as determined by ^{3}H-colchicine binding assay, while that of the sham-operated animals showed no change. Total tubulin content did not change in either OVX or sham animals. The polymerized pool and the total tubulin in 9-day lactating mammary glands were significantly greater than in the mammary glands of OVX or sham rats.

Three types of evidence support the involvement of microtubules in milk secretion. First, colchicine and other microtubule-altering drugs inhibit the secretion of specific milk components including fat (1, 2), protein (1–3) and lactose (4, 5). *In vivo* retrograde administration of these drugs via the milk ducts inhibits milk flow in goats (6). Second, a morphological association of microtubules with the secretory apparatus has been established by electron microscopic studies showing a vectorial increase in the number of microtubules from basal to apical poles (7) and by immunofluorescent studies which demonstrate a similarly increased concentration of tubulin protein in the apical cytoplasm of lactating alveolar cells (8). Finally, in guinea pig mammary gland the pool of polymerized (microtubular) tubulin increases severalfold from late pregnancy to peak lactation (9). Immunofluorescence studies (8) confirm a lactogenesis-related increase in alveolar cell tubulin.

These observations suggest that increased microtubule formation may be included in the lactogenic response of the mammary gland. To test this possibility, premature lactogenesis was induced in late pregnant rats by removing both ovaries and measuring free and polymerized tubulin pools in the mammary glands at various time intervals following ablation, using a ^{3}H-colchicine binding assay. In this manner changes in both the total tubulin concentration as well as the relative size of the polymerized fraction could be observed. Animals in peak lactation were also studied for comparison.

Materials and Methods. *Animals.* Sprague–Dawley 13-day sperm-positive rats were obtained from Holtzman Rat Company (Madison, Wisc.) and maintained on a 14:10-hr light:dark cycle (lights on at 0500 hr). Food (Purina Laboratory Chow, Ralston Purina Co.) and water were provided *ad libitum.* On Day 18 of pregnancy most animals were subjected either to bilateral ovariectomy (OVX) through two dorsolateral incisions or sham OVX under light ether anesthesia. Surgery was performed at various times of the day, but animals were always killed and tissue samples obtained at 0900–1100 hr at 6, 12, 18, 24, 36, and 48 hr after surgery. Six nonoperated, 18-day pregnant animals were used to obtain control data, and peak lactation values were obtained from six 9-day nursing rats.

Sample preparation. Rats were killed by cervical dislocation and decapitation. Blood was collected immediately through a cheesecloth filter for progesterone assays. Inguinal and abdominal mammary glands were quickly removed, trimed of visible fat and connective tissue, cut into small fragments with razor blades, rinsed with 40 m*M* TRIS–HCl buffer (pH 7.4) and damp-dried on filter paper. One aliquot was weighed and homogenized (5% w/v) in 10 m*M* TRIS–HCl buffer (pH 7.4) with 2 m*M* EGTA and used for lactose and protein assays. A second aliquot of tissue (about 450 mg) was weighed and homogenized (15% w/v) in microtubule stabilizing buffer (MTS) for the tubulin assays. MTS is composed of 50%

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glycerol, 5% dimethyl sulfoxide, 0.5 m*M* EGTA, 0.5 m*M* $MgCl_2$, 10 m*M* phosphate buffer (pH 6.95), and 0.5 m*M* GTP.

Assays. The free and polymerized (microtubular) tubulin pools were assayed by a modification (10) of the ^{3}H-colchicine binding of Pipeleers *et al.* (11). Free tubulin dimers and polymerized microtubules were separated in two steps. The tissue was homogenized in glycerol-containing MTS buffer which yielded free tubulin in the 100,000*g* supernatant at room temperature; the microtubules in the pellet were then disassembled in a second, tubulin-solubilizing (TS) buffer (0.25 *M* sucrose, 0.5 m*M* $MgCl_2$, 10 m*M* PO_4 buffer (pH 6.95), and 0.5 m*M* GTP) in the cold and a second 100,000*g* centrifugation at 0–4°C yielded endogenous microtubule tubulin now in the dimer state. Both fractions were incubated with ^{3}H-colchicine and the amount of bound colchicine taken as a measure of tubulin. The latter can be expressed either as total tubulin (free + polymerized) or percentage polymerized tubulin.

The following modifications were introduced into the assay in adapting it to mammary gland: The concentration of ^{3}H-colchicine bound during incubation was 42 μM and 11 μM, respectively, for the MTS and TS buffers. Incubation times were 4 hr for MTS and 1 hr for TS. Separation of bound and unbound ^{3}H-colchicine was carried out on 180 × 8-mm Sephadex G-100 columns rather than charcoal.

Lactogenic indices. Progesterone was determined by radioimmunoassay in blood-serum samples extracted with hexane and evaporated under N_2 at 37°C (12). Lactose was assayed using the β-galactosidase–galactose dehydrogenase method utilizing NAD reduction. Protein was measured by the method of Lowry (13).

Results. *Lactogenic parameters.* Progesterone blood levels in the sham-operated animals fell gradually from 160 ng/ml plasma to 56 ng/ml 48 hr after surgery (i.e., Day 20 of pregnancy). Ovariectomy caused progesterone levels to fall to 27 ng/ml by 6 hr and remain at less than 5 ng/ml through 48 hr. Those of the lactating animals averaged 66 ng/ml. Lactose concentrations in the mammary glands of sham and OVX rats were similar through 18 hr after which the lactose levels of the OVX rats rose from about 0.5 μg/mg tissue to 3.4 μg/mg (700%) by 24 hr and to 5.9 μg/mg (1240%) by 36 hr. Lactating tissue contained only 2.5 μg/mg; however, these animals were actively secreting milk and delivering lactose to the pups. Mammary-gland protein remained constant in both sham and OVX rats (approx. 5.5 mg %) through 12 hr after which protein from the sham rat slowly rose to 6.5 mg % while that from the OVX rat rose to 8 mg %.

Tubulin changes. The total tubulin concentration in mammary glands of six, intact, 18-day pregnant rats was 10.4 ± 2.2 pmole/mg protein ($\bar{X}$ ± SD) of which 3.6% was in the polymerized state (Fig. 1). Tubulin concentrations in the sham and OVX rats' mammary glands were similar during the 48-hr period following surgery (14.7 ± 3.8 and 14.3

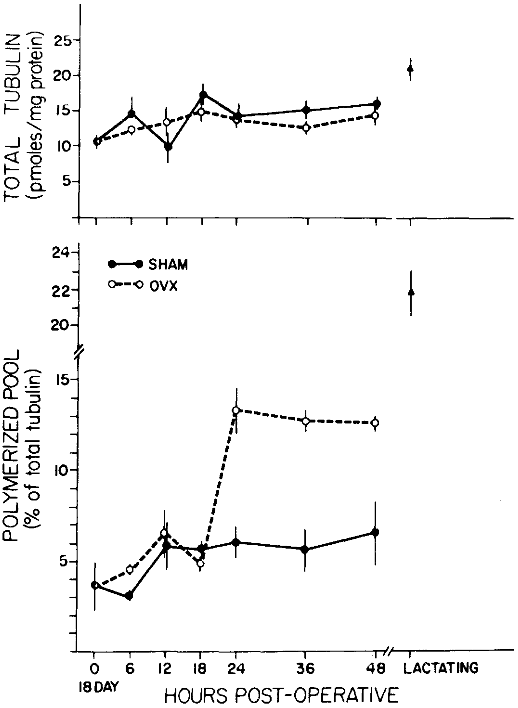

FIG. 1. Changes in rat mammary gland tubulin following bilateral ovariectomy (OVX). Upper graph: total (free + polymerized) tubulin content based on ^{3}H-colchicine binding assay. Lower graph: fraction of total tubulin in polymerized (microtubular) state. OVX values are compared to sham-operated and 9-day lactating (▲) values. Usually, each point is the mean of ±SEM of six or more animals (3 at 48 hr).

± 3.7 pmole/mg protein, $N = 33$ and 41, respectively), however, the six lactating rats' mammary glands contained 21.0 ± 3.9 pmole tubulin/mg protein. This was significantly greater ($P < 0.01$) than the values obtained by averaging those from samples taken at 24, 36, and 48 hr for both the sham (15.0 ± 3.5 pmole/mg) and OVX (13.4 ± 2.9 pmole/mg) animal.

The values of the polymerized pool of the sham and OVX animals during the first 18 hr following surgery (4.7 ± 2.1% and 5.4 ± 2.5%, respectively) are not different and not different from the 18-day intact animals although the means of the sham and OVX rats are about 40% higher than the intact. Between 18 and 24 hr, however, the OVX values increase abruptly while the sham values remain the same. Mean values for the time period 24 through 48 hr are 13.0 ± 3.1% for the OVX and 6.0 ± 2.6% for the sham. The polymerized-pool mean value in the lactating rat mammary glands was 21.8 ± 3.1% which is significantly different ($P < 0.001$) from both the sham and OVX rat mammary gland 24- to 48-hr values.

Discussion. This study has demonstrated that, following ovariectomy, although the concentration of tubulin protein in mammary gland is not altered, the fraction of tubulin in the polymerized state rises severalfold after a lag period of about 18 hr. That this increase may be a normal lactogenic event is suggested by the fact that the polymerized pool in the lactating gland is also high, nearly twice that of the OVX group.

The results are in agreement with previous studies from this laboratory using guinea pigs. First, the percentage polymerized tubulin found in mammary gland biopsies rose sevenfold from 1 week prepartum to midlactation and then decreased toward weaning (9). In an immunofluorescence study, specific antitubulin staining was not evident in late pregnant mammary gland sections, but sections from peak lactating glands showed strong apical fluorescence of all alveolar cells. In early lactation (24–48 hr), glands demonstrated staining intermediate between pregnant and peak lactating glands (8). Finally, in cell populations isolated from late pregnant and lactating guinea pig mammary glands, alveolar cells contained twice as much tubulin as other cell types and the fraction of tubulin in the polymerized state was also twice that of other cells (14).

One area in which reproductive endocrine influences on microtubules has been studied is that of ciliogenesis in the oviduct. In species as diverse as the quail (15), the monkey (16), and the cat (17), differentiation of oviduct epithelium, including promotion of ciliogenesis, is stimulated by estrogen. Other studies suggest an inhibitory influence by progesterone (18). Estrogen stimulation in this study is unlikely in view of the effects of ovariectomy. The analogy between ciliary microtubules and cytoplasmic microtubules that could participate in secretion or intracellular transport is tenuous considering the complexity of cilia and the stability of their microtubular infrastructure. In addition, the effects of estrogen on ciliogenesis involved several phases including the formation of procentrioles, basal bodies, ciliary buds, etc. Such large-scale changes in cytoarchitecture suggest a significant increase in the synthesis of tubulin and associated proteins rather than a simple shift in the equilibrium of the tubulin pool toward microtubules. In the present study, accelerated tubulin synthesis does not seem to occur. One common point is the inhibitory actions of progesterone. It is inhibitory to ciliogenesis, and its withdrawal is thought to act as a trigger for lactogenesis in mammary gland (19). A similar effect has been observed with cat uterus myosin filaments of which estradiol is reported to promote assembly and progesterone disassembly (20). Several factors are known to affect microtubule assembly *in vitro:* critical concentration of tubulin dimers, GTP, high-molecular-weight microtubule-associated protein, Ca^{2+}, cyclic nucleotides, and temperature (21). Further studies are required to determine if the hormonal changes induced by ovariectomy affect any of these factors which may help to elucidate the mechanism for accelerated microtubule assembly during lactogenesis.

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