Estrogen Action at Endometrial Membranes: Alterations in Luminal Surface Detectable within Seconds

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ABSTRACT The morphological effects of estrogen on the luminal surfaces of rat endometrial cells were investigated by scanning electron microscopy. Ovariectomized rats were injected intravenously with estradiol-17β (E₂β), 0.5 μg/0.25 ml per 100 g body wt. At various intervals thereafter, the lumen of a uterine horn was flushed with buffered 2% glutaraldehyde and then prepared for scanning electron microscopy by conventional methods. In control rats that had received an equivalent volume of placebo vehicle, the luminal cell surface was characterized by short, sparse microvilli (MV) and, in most cells, a single, central cilium. At 30 s after E₂β injection, the number of MV was significantly increased. By 1 min, MV density was further increased and MV were frequently clustered; also, the central cilium of many cells was no longer evident. Similar results were obtained after exposure to diethylstilbestrol for 30 s to 1 min, whereas neither a subthreshold dose of E₂β nor a dose of the relatively inactive congener E₂α equivalent to a saturating concentration of E₂β gave statistically significant responses in surface changes by the present criteria.

After 3–7 min of E₂β exposure, MV had increased greatly in length and density. These effects underwent dramatic regression by 15–30 min after E₂β treatment, with distinct diminution of microvillar lengths and numbers, reduction of clustering, and reappearance of the central cilium in many cells. This was succeeded at 1 h by a renewed surge of surface activity. These results are consistent with cumulative evidence for rapid alterations of the surface membrane of estrogen-sensitive cells in response to physiological levels of active hormone. Whether these responses in the luminal surfaces are primary, or are secondary reflections of receptor-mediated membrane alterations at the basolateral blood-front, remains to be determined.

The influence of ovarian hormones on endometrial morphology has been studied for over two decades with the use of both transmission, and, more recently, scanning electron microscopy (SEM). It is now well established that the endometrial surface undergoes a variety of structural alterations in cyclic rats (1, 2), humans (3–7), and other mammals (8, 9), and that these modifications are under the dual control of estrogen and progesterone. Changes in endometrial ultrastructure have also been shown in ovariectomized rats in response either to a single dose (10) or to daily doses (2) of estrogen. Presumably on the basis of the assumption that these changes were mediated by estrogen-induced proteins, the times chosen for observation have ranged from 4 h to many days after hormone administration, although most observations have been made after 12–48 h.

However, cumulative evidence (reviewed in reference 11) has revealed that numerous signals attributable to perturbation by estrogen of the surface membranes of its cellular targets occur within mere seconds to minutes of administration of this hormone to ovariectomized rats, thus greatly preceding the relatively delayed morphological indicators cited above. Accordingly, in the present study, we have used SEM in an attempt to detect early correlates of estrogen action on endometrial surface morphology. The present work demonstrates that such effects, which are confined to estrogenually active compounds, were indeed evident within seconds to minutes of the intravenous administration of physiological levels of active estrogen.
MATERIALS AND METHODS

Preparation of Steroid Solutions: Estradiol-17β (E2β), estradiol-17α (E2α), and diethylstilbestrol (DES) were freshly prepared from ethanolic stock solutions on the day of experiment and maintained at 0°C until brought to room temperature just before use. Concentrations of these were verified spectrophotometrically at intervals in the progress of the work, using established absorption coefficients (12). The appropriate aliquot of stock solution was pipetted into a small flask and evaporated to near-dryness under a stream of nitrogen. The residue was dissolved in that volume of Dulbecco’s PBS (Gibco Laboratories, Grand Island, NY) which yielded the required maximum concentration of hormone. Dilutions were made with vehicle to permit administration of equivalent volumes at all dose levels.

Experimental Procedures: Female Sprague-Dawley rats were ovariectomized at 6 wk of age and kept in a low-steroid environment for 3 wk, as previously described (13). Careful maintenance of the low-steroid environment was found to be crucial to the success of these experiments.

The animals were anesthetized lightly with subcutaneous Nembutal (sodium pentobarbital; 5 mg/100 g body wt). 15 min later, they were injected by saphenous vein either with hormone in doses ranging from 0.05 to 0.5 μg/0.25 ml per 100 g body wt or with equivalent volumes of vehicle alone. After selected intervals, the right uterine horn was sectioned ~2 mm from the cephalic end, to assure patency for flushing. It had been established in preliminary experiments that the two cornua gave equivalent responses. A 2% (vol/vol) solution of EM grade glutaraldehyde (Polysciences, Inc., Warminster, PA) in 0.1 M phosphate buffer (pH 7.2) was injected into the lumen with a 27-gauge needle, inserted through the horn just above its junction with the corpus. The lumen was gently flushed with ~0.4 ml of fixative, then severed near the corpus. Mesometrial fat was rapidly removed with fine scissors, leaving the major vessels intact. The horn was cut in half longitudinally and then into 2-3-mm strips and fixed by immersion in the buffered glutaraldehyde for 1 h at room temperature. The specimens were then washed twice for 10 min each time in phosphate buffer and dehydrated in the standard series of ethanol solutions. Critical-point drying was carried out in a Samdri PVT-3 instrument (Tousimis Research Co., Rockville, MD). The samples were mounted, coated with gold-palladium using a Hummer 1 coater (Technics, Inc., Alexandria, VA), and viewed with a scanning electron microscope (Etec; Hayward, CA).

For each type of experimental regimen, between two and six experiments were performed. Micrographs were taken at random, at magnifications ranging from 1,000× to 20,000×.

Selection of Time Points: The earliest time point chosen for observation was 30 s, which in the rat encompasses five circulation times (14). As steroid uptake is essentially instantaneous (15), this interval was deemed adequate to permit substantial capture of the steroid by target cells. Other times for observation were chosen on an expanding time scale.

Quantification of Microvillar Density: All micrographs used for quantification were made at x 8,000 and used directly, without photographic enlargement. Microvilli (MV) within a 4-cm² area in the center of each micrograph were counted blind, on two separate occasions. The data from the several experiments of the same type were pooled. In accord with the available numbers of micrographs (from 6 to 19) for each variable, n represents the individual observations. Statistical comparison of the results was obtained with the Student’s t test.

RESULTS

Acute Effects of E2β

The luminal surface of endometrium from two control animals, which received vehicle alone, is shown in Fig. 1, A and B. Cells with well-defined borders were characterized by short, sparse MV. Many cells also displayed a single central cilium.

The luminal surface changed very rapidly in response to estrogen. By 30 s after a physiological dose (0.5 μg/100 g body wt) of E2β, MV were manifestly increased in number (Fig. 1E). Statistical analysis revealed that the increase in MV density attributable to estrogen exposure in vivo was significant by 30 s (P<0.001; n=28).

Progressive Nature of the Morphologic Changes

The estrogen-induced response was progressive, as evidenced by a further increase in MV number by 1 min after E2β injection (Fig. 1F). At this time, MV density was significantly greater than that seen at 30 s (P<0.001; n=23). The increased density of MV on the surfaces of endometrial cells within 30 s to 1 min of E2β administration, as summed from a series of separate experiments, is shown graphically in Fig. 2.

In many cells, the MV appeared to be somewhat longer by 1 min after E2β and occurred in clusters (Fig. 1F). The latter phenomenon was never seen in control preparations nor in those observed after only 30 s of E2β action. Also evident by 1 min was the disappearance from many cells of the central cilium (cf. Fig. 1F vs. Fig. 1A). The intercellular borders were thicker and more densely invested with MV in these preparations than in those from controls.

By 3 and 5 min after E2β, MV were noticeably increased in length beyond the dimensions seen at 1 min (Fig. 3A). These longer MV were often partially superimposed on each other, preventing accurate MV counts; therefore, density data for these time points were not included in Fig. 2. This was also the case for samples that had been exposed to E2β for 7 min (Fig. 3B). At this time, MV of many cells were very densely concentrated, giving the appearance of a thick mat. Moreover, in the samples representing the interval of 1-7 min of E2β action, there was much variation in degree of responsiveness of individual cells. As is well illustrated in Fig. 1F, cells with sparse MV and a prominent cilium were often found adjacent to cells with dense, clustered MV and with no apparent cilium. Cell-to-cell variation in estrogen sensitivity has long been recognized (11, 16).

Tuft-like structures, which occurred at the junction of a group of five or six cells, were distributed sporadically on the luminal surface (Fig. 3, C and D). Such tufts were numerous in estrogen-treated samples and were very rarely found in control samples.

Dose-Response Relations

Although the dose of E2β used in the experiments described above (0.5 μg/100 g body wt) is considered to be within the physiological range, a dose of only 0.1 μg/100 g body wt has been shown, by some criteria, to be adequate for maximal stimulation (17). Therefore, we investigated the effects on luminal surface morphology of a subthreshold dose of E2β which has been shown to be ineffective in eliciting adenylate cyclase stimulation at times concurrent with those presently under investigation (17). Fig. 3E illustrates the lack of surface effects of 1 min of exposure to a subthreshold dose of 0.05 μg/100 g body wt. The appearance of such preparations was indistinguishable from that of controls (Fig. 1, A and B). However, in preliminary experiments, a 1-min exposure to 0.1 μg of E2β/100 g body wt elicited a degree of surface activity appreciably enhanced over the control level (not shown).

Structural Specificity of Agonist

The synthetic estrogen DES, at a dose of 0.5 μg/100 g body wt, had an effect similar to, but not identical with, that of E2β, as seen in Fig. 3F. Thus, although by 1 min after DES treatment many cells in dense clusters similar to those seen after 1 min of E2β exposure, many MV in the DES series appeared substantially longer than those seen in corresponding E2β-treated samples, suggestive of a more advanced response.

In contrast, E2α had only a limited effect on luminal surface morphology. This epimer, which has been shown to be approximately 1/40–1/100 as effective as E2β by most criteria.
FIGURE 1 Scanning electron micrograph of the luminal surface of uterine endometrium from an ovariectomized rat (A) 1 min and (B) 30 s after intravenous injection of vehicle alone. Cell boundaries, short MV, and central cilia are evident. Bar, 0.25 μm × 4,000. (C) High magnification view of a cilium from a control sample. A cell boundary can also be seen. Bar, 1 μm × 16,000. (D–F) Luminal surfaces of endometrium, showing acute effects of exposure to 0.5 μg/100 g body wt of E2. (D) 30 s after E2, high magnification view. MV have become more numerous. Bar, 1 μm × 16,000. (E) 30 s after E2, lower magnification. Bar, 0.25 μm × 4,000. (F) 1 min after E2. Note clustered MV, absence of central cilium on some cells, and a degree of variation in responsiveness from cell to cell. Bar, 0.25 μm × 4,000.
very rapid structural changes in the luminal surfaces of estrogen treatment for 1 h could be distinguished qualitatively combined features, the surface stimulation attributable to short and somewhat irregular in shape, although an occasional

dominal cells in response to active estrogens. These alterations included increased MV density and length, clustering of MV, disappearance of central cilia, and appearance of tuft-like structures at cell junctions. These changes, which were detectable within 30 s of the intravenous administration of a single physiologic dose of the hormone to ovariectomized rats, were intensified in degree and complexity at 1–7 min after estrogen, and were diminished by 15–30 min. Indeed, but for the appearance of an occasional rounded protuberance (“bleb”), the specimens examined at 30 min after the hormone, with the profoundly shortened and relatively diminished numbers of MV, together with the reemergence of central cilia in most cells, closely resembled preparations fixed at intervals from 30 s to 60 min after injection of control vehicle. However, by 1 h after E2β, a secondary surge of surface activity became evident, indicating that the architectural modifications elicited by estrogen are biphasic.

There is mounting evidence that steroid hormones, including estradiol, are, like peptidal effectors, subject to recognition by macromolecules intrinsic to the surface membranes of their cellular targets (19–21). By a number of criteria, these moieties conform to the properties requisite to specific receptors (21). Moreover, as documented in some detail for estrogen, the array of biochemical and biophysical signals generated by such surface interaction is generally associated with plasmalemmal perturbation evident by 15 s to 2.5 min (11). The onset of the presently described alterations in endometrial surface morphology was thus of an order similar to that of other rapidly induced estrogenic responses. To our knowledge, these are the earliest estrogen-induced structural changes yet reported, although in preliminary reports, estrogen has been shown to elicit enhancement of micropinocytotic activity in the uterus within 2 min of its intravenous injection to ovariectomized rats (16, 22). However, several of the features we describe have also been identified at later times after estrogen injection. For example, clumped MV similar to those in Fig. 1 F have been observed by Plapinger (23) in the vaginal epithelium of mice treated with DES for 5 d. Also, the tuft-like structure in Fig. 3 C has been reported by Anderson et al. (2) in the uterus of a cyclic rat. Many authors have noted an expansion of MV number and length in response to exogenous estrogen. In rat uterus, this phenomenon was seen within 12–24 h after subcutaneously administered estrogen injection (2); as noted, no earlier observations appear to have been made (2). Our data indicate that the MV development described at these later times is secondary to an initial, but astonishingly transitory, increase within seconds of estrogen treatment. The underlying basis for the biphasic nature of rapidly evolving surface events here described is obscure. However, it is possible that the biochemical events accompanying the increased surface activity, such as augmented pinocytosis, increased plasmalemmal permeability to ions and water, and enhanced liberation of lysosomal hydrolases (13, 24), with concomitant increases in osmotically active particles, may, in concert, result in increased cellular turgor, thus temporarily overwhelming the tendency toward surface extensions. The balance in favor of net increases in surface membrane complexity may then once more be restored through redistribution of ions and the onset of net anabolic reactions engendered by the hormone, with remanifestation of surface activity.

In addition to the several features of luminal MV in response to active estrogen, our experiments also reveal hor-

**Figure 2** Enumeration of uterine MV at very early intervals after intravenous injection of control vehicle, active estrogen (E2α), and its relatively inactive congener (E2β). The height of the bars presents the mean number of MV counted under standardized conditions (see text) in each of 6–19 representative sections of the specified control and steroid-injected specimens. Variations shown are standard error of the mean in 2–6 separate experiments.

(c.f. reference 18), elicited only a small increase in MV density by 1 min of the intravenous administration of 0.5 μg/100 g body wt (Figs. 2 and 4A); this was without statistical significance (0.4 > P > 0.3; n = 21). Generally, the surface features of the E2α-treated cells resembled those of the vehicle-treated controls, with sparse MV and a distinct central cilium (cf. Fig. 1, A and B). In a limited number of experiments, the inert sterol cholesterol, at a dose of 0.5 μg/100 g body wt, had essentially no effect when compared with parallel vehicle controls (not illustrated).

**Biphasic Nature of the Response to Active Estrogen**

As shown in Fig. 4, B and C, the acute effects of E2β on the morphology of the endometrial surface were transitory. By 15 min after E2β (Fig. 4B), MV density was less than that seen at 7 min (Fig. 3B), although elongated MV were present on some cells, as were clustered MV. By 30 min, both of these phenomena were rarely seen (Fig. 4C). MV were sparse, short, and scattered. The central cilium once again became conspicuous.

Surprisingly, by 1 h after E2β the luminal surface was again densely covered by MV (Fig. 4D). In general, the MV were short and somewhat irregular in shape, although an occasional long MV could be identified. Some cells also displayed bleb-like structures, larger and more rounded than MV. By these combined features, the surface stimulation attributable to estrogen treatment for 1 h could be distinguished qualitatively from the responses characteristic of E2β action at 1–7 min.

**DISCUSSION**

By means of SEM, the present studies provide evidence of very rapid structural changes in the luminal surfaces of en-
FIGURE 3  (A) Luminal surface 5 min after E₂β. Many elongated MV are apparent.  (B) 7 min after E₂β. MV occur in dense patches.  Bar, 0.25 μm. × 4,000.  (C–D) Tuft-like structures at junction of several cells 5 min after E₂β.  (C) Bar, 1 μm. × 16,000.  (D) Bar, 0.25 μm. × 4,000.  (E) Luminal surface 1 min after intravenous injection of 0.05 μg/100 g body wt of E₂β. This dose of estrogen was ineffective in eliciting the degree of surface changes seen in Fig. 1f.  Bar, 0.25 μm. × 4,000.  (f) 1 min after exposure to 0.5 μg/ 100 g body wt of DES.  Elongated MV have formed clusters similar to those seen at 1 min following injection of E₂β (Fig. 1f).  Bar, 0.25 μm. × 4,000.
Figure 4  (A) 1 min after exposure to 0.5 μg/100 g body wt of the relatively inactive epimer, E₂α. Luminal surface is similar to control (Fig. 1A), with sparse, short MV. (B-D) Later effects of 0.5 μg/100 g body wt of E₂β. (B) 15 min after E₂β. MV density is less than at 7 min (cf. Fig. 3B). More central cilia are apparent than occur between 1 and 7 min (cf. Figs. 1F and 3A). (C) 30 min after E₂β. As at 15 min, MV are diminished in length and number. (D) 1 h after E₂β. Luminal surface has become covered with irregularly shaped MV and small blebs. Bars, 0.25 μm. × 4,000.

Estrogen-induced changes in the frequency of luminal surface ciliation. The solitary central cilia seen on many cells in our control samples have been previously observed in ovariectomized rats by Tachi et al. (10). These structures differ from motile cilia in that they possess a "9 + 0" microtubular arrangement and are associated with diplosomes. Cilia of this type have been observed on epithelial cells in vivo (25-31) and also on some cultured cells (32, 33). Using the 3T3 cell line, Tucker et al. (34) demonstrated that the cilium is "lost" when a cell initiates DNA synthesis and that the centriole is reciliated following the completion of mitosis. Similarly, Rieder et al. (35) found that the cilium of PtK₂ cells is gradually resorbed during spindle formation. Although the specific function of the central cilium is as yet unknown, these studies indicate that its disappearance is closely related to the initial events in cell division.

Tachi et al. (10) have shown that ciliary loss by rat endometrial cells occurs by 12 h after a single injection of estrogen, the earliest observation time reported. However, the present work demonstrates that on many cells the cilium was lost within 1 min of estrogen treatment. By the present criteria, it was not possible to discern whether the cilium had been retracted, shed by a form of exocytosis, or simply obscured by the clustered MV. Moreover, the rapidity of restoration of this organelle (30 min after a single intravenous dose of E₂β) is likewise enigmatic. Further investigation will be necessary to clarify the relationship of these early alterations in ciliary dynamics to the cell division, which is known to occur 24-48 h later.

Estrogen is not unique in its induction of rapid structural changes at the surface membrane or cortical cytoplasm of target cells. Connolly et al. (36), using SEM, have observed surface ruffles in cultured sympathetic neurons by 30 s after nerve growth factor; these become prominent by 1 min, and disappear by 7 min. Similarly, in the human carcinoma cell line A-431 incubated with epidermal growth factor, large lamellipodia are formed within 5 min of the addition of growth factor but are withdrawn 5-15 min later (37). It is significant that in both of these examples, the biphasic surface changes were induced by peptide growth factors for which
there are known to be surface receptors. Our present work demonstrates that estrogen, a steroid hormone, triggered similar changes in a target organ, a finding that is consistent with biochemical and ultrastructural evidence of the occurrence of steroid hormone receptors in the plasma membrane (11). There are growing indications for parallels in many additional features of the sequential responses to the two structurally dissimilar classes of effector (13, 16, 38).

The rapid but transitory alterations in the cellular architecture here described may be coupled, in some manner presently unknown, to later hormone-induced responses. It is tempting to suggest, on the basis of indications already available, that information associated with deformation of the cell surface on ligand recognition is communicated inward by some component of the cytoskeleton (13, 22, 39). Clearly, such a complex problem will require extensive further investigation.

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