ESTROGEN-INDUCED MEMBRANE ALTERATIONS
AND GROWTH ASSOCIATED WITH PROTEINASE ACTIVITY
IN ENDOMETRIAL CELLS

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ABSTRACT

Endometrial cells isolated from uteri of ovariectomized rats were treated in vitro with $1 \times 10^{-9}$ M estradiol-17β ($E_2\beta$) to analyze early changes in membrane properties during hormone-induced growth. After 30-min exposure to $E_2\beta$ at 22°C, cells exhibited an enhanced capacity to bind erythrocytes (hemadsorption) in the presence of concanavalin A (Con A) to 237% of the level in paired controls. Fluorescence microscopy revealed that ~25% of cells exposed to $E_2\beta$, but not estradiol-17α ($E_2\alpha$), showed a redistribution into polar clusters of Con A-binding sites that were dispersed in random patches at the external surfaces of control cells. These hormone-induced membrane alterations were abolished by prior treatment of cells with inhibitors of thiol proteinase activity of the cathepsin B1 (CB1) type, such as leupeptin and iodoacetate. Leupeptin at $4.5 \times 10^{-7}$ M also reduced the affinity of [α-3H]E2 binding to intact cells but did not influence specific binding of the hormone to macromolecular components of cytosol. A pronounced increase in the availability of endogenous CB1, but not of alkaline phosphatase, succinate, or lactate dehydrogenase, in the extracellular media was elicited within 30 min after $E_2\beta$ treatment. In cells cultured in chemically defined medium for up to 48 h, $E_2\beta$, but not $E_2\alpha$, enhanced cell proliferation and stimulated [3H]thymidine incorporation into macromolecular form. These $E_2\beta$-induced effects were abolished by prior treatment of cells with liposome-entrapped leupeptin at a final concentration of $7 \times 10^{-8}$ M. The net rate of intercellular adhesion among endometrial cells was also enhanced by $E_2\beta$. This hormonal response was diminished by prior exposure to leupeptin. Fractionation of cells by selection for adhesiveness due to $E_2\beta$ exposure for 30 min yielded a subpopulation of rapidly dividing cells which surpassed their less adhesive counterparts in cathepsin secretion and in Con A-mediated hemadsorption. These results indicate that leupeptin-sensitive proteinase activity may contribute to membrane and growth modifications elicited by $E_2\beta$ treatment in endometrial cells.

KEY WORDS estrogen • endometrial cells • intercellular adhesion • concanavalin A • cell proteinase • leupeptin • cathepsin • surface
Specific binding of estradiol-17β (E2β) to extranuclear macromolecules is well documented (cf. reference 18). These receptor components function in transfer of the hormone to the nuclear compartment, where the complex appears to promote expression of the phenotypic effects (8, 19, 23, 25). Additional data indicate that limited hydrolysis of the native receptor by a Ca2+-activated proteinase occurs concomitant with the formation of the mobile estrogen-binding derivative which enters the nucleus (47, 52). Moreover, estrogen-induced translocation of Ca2+ which enters the nucleus (47, 52). Moreover, estrogen-induced translocation of Ca2§ to the nuclear compartment of target cells has also been reported.

Mild treatment of normal cells with exogenous proteinases is known to lead to alterations in the cell surface (cf. references 9 and 33) and a level of cell growth (cf. references 9, 10, and 33) that are usually seen only in transformed or tumor cells. Moreover, changes in lectin-mediated agglutinability (42), Ca2+ exchange (40), and proteinase availability (43) at the surface membranes of isolated endometrial cells have been detected shortly after their exposure to estrogens in vitro. In turn, enhanced mitotic activity of endometrial cells 24 h after estrogen treatment (15) is closely correlated with increased thymidine incorporation into DNA (17, 28). Preliminary findings indicate that enhancement of DNA synthesis and the formation of the mobile estrogen-binding derivative of target cells has also been reported.

At the start of most experimental manipulations, the intact cells were suspended in Ringer's solution composed of 136.9 mM NaCl, 2.7 mM KCl, 0.5 mM CaCl2, 0.5 mM MgCl2, 1 mM sodium pyruvate and buffered at pH 7.4 with 8.1 mM Na2HPO4 and 1.5 mM KH2PO4. Essentially all vessels used for these preparations were polyethylene, polycarbonate, or polypropylene. All materials and instruments used in these procedures were obtained sterile or autoclaved, and all solutions were filter-sterilized.

**Determination of [3H]E2β Binding by Intact Cells and Their High-Speed Supernatant Fractions**

Levels of estradiol binding in intact cells were determined by methods described previously (44). In brief, endometrial cells were suspended in Ringer solution to a final concentration of 2 x 107 cells/ml. Incubations of 30 min duration were begun with the addition of 1 x 10-8 M [2,4,6,7-3H]E2β (99 Ci/mmol; New England Nuclear, Boston, Mass.) at 22°C in an atmosphere of 100% O2 and with shaking at a rate of 90 oscillations/min. A 200-fold excess of unlabeled E2β (Schering Corp., Kenilworth, N.J.) was added to paired samples for determination of displaceable binding (cf. reference 62).

High-speed supernatant (i.e., cytosol) fractions were prepared from freshly isolated endometrial cells at 4°C by slight modification of the method of Jensen et al. (23). In brief, intact cells were homogenized in 4 vol of 10 mM Tris-HCl buffer (pH 7.4) with 1.5 mM disodium EDTA. Approximately 15 manual up-and-down strokes with a glass-glass homogenizer were sufficient to disrupt the endometrial cells as judged by phase-contrast microscopy. The homogenate was diluted with an equal volume of buffer and filtered twice over two layers of nylon mesh with an average pore size of 35 μm (Niton; Tobler, Ernst, and Traber, Inc., Elmsford, N.Y.). The filtrate was centrifuged at 105,000 g for 1 h. The particle-free supernate was collected and incubations were begun with the addition of 1 x 10-8 M [3H]E2β at 4°C. A 200-fold excess of unlabeled E2β was present in paired samples for determination of displaceable binding. An incubation time of 2 h was used in these experiments to allow sufficient time for the binding process to achieve equilibrium at 4°C. After 2 h, the free estradiol was removed by the dextran-coated charcoal procedure (26), and aliquots of the samples were analyzed for bound [3H]E2β by liquid scintillation counting.
Cell Proliferation, DNA Content, and Incorporation of \[^{3}H\]Thymidine into Macromolecular Form in Endometrial Cell Cultures

Isolated endometrial cells were cultivated in serum-free, chemically defined medium (CDM) in a humidified atmosphere of 5% CO\(_2\) in air at 37°C. CDM consisted of Earle's balanced salt solution enriched with 1 mM dextrose, 1 \times 10^{-4} M highly purified crystalline bovine insulin (Eli Lilly and Co., Indianapolis, Ind.), 0.1% (wt/vol) albumin (Pentex), Modified Eagle's Medium amino acids adjusted to physiological concentrations (Grand Island Biological Co., Grand Island, N. Y.), and Gentamicin (50 µg/ml; Schering Corp.). After 2-d incubation, the CDM was removed and endometrial cells were washed with 2 ml of fresh solution. 5 ml of new CDM with or without test compounds was then added to each dish. Incorporation of \[^{6}H\]thymidine (NET 355; 10.5 Ci/mmol; New England Nuclear) into acid-precipitable form from cell cultures after 20-h incubation was measured as described previously (38).

In independent experiments, proliferation of endometrial cells after 24- or 48-h incubation was determined from cell numbers. Cells were suspended in Ca\(^{2+}\)-, Mg\(^{2+}\)-free Ringer's solution in the presence of 1.5 M EDTA and counted in a bright-line double Neubauer counting chamber using phase-contrast optics. In 25 separate experiments, the cell numbers over a range of values from 1.5 to 5.0 \times 10^6 showed a positive linear correlation (r = 0.94) with DNA levels in paired samples, as determined by the method of Hill and Whatley (20). The DNA concentrations in these experiments averaged 8.4 \pm 0.2 µg/10^6 endometrial cells.

Concanavalin A Binding and Concanavalin A-Mediated Hemadsorption to Isolated Endometrial Cells

Specific binding of 100 µg concanavalin A (Con A; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.)/ml to endometrial cells was determined with fluorescein-labeled Con A (F-Con A) as described by Pietras and Szego (42). Assessment of Con A-mediated adsorption of homologous erythrocytes to isolated endometrial cells (i.e., hemadsorption) was carried out as previously published (42).

Dark-Field Ultraviolet Fluorescence Microscopy

Photomicrographs were obtained with a Leitz Ortholux microscope, using a xenon light source, XBO 150. The exciting filter combination consisted of a BG 38 heat-barrier filter, a Leitz 480 nm filter, and a Leitz KP 490 interference filter; a K 510 nm filter served as the barrier filter. Distribution of fluorescein label was analyzed after thorough washing of preparations (1.5 \times 10^6 cells/ml) that had been treated with 100 µg F-Con A/ml for 3 min at 22°C as described previously (37, 42).

Analyses of Extracellular Hydrolase Activities

Suspended endometrial cells (approx. 6 \times 10^6 cells/2 ml Ringer's solution) were incubated with or without additives for selected times in a Dubnoff shaking incubator at 22°C. The cells were then sedimented by centrifugation at 400 g for 5 min at 4°C in a Sorvall RC2-B centrifuge (DuPont Instruments, Wilmington, Del.). Particle-free supernatant fractions of the incubation media of isolated cells were then obtained by further centrifugation as described previously (42), and samples of these were collected for analyses of hydrolase activities.

Cathepsin B1 (EC 3.4.22.1; CB1) activity was determined by procedures described by Szego et al. (59), using carbobenzyloxy-alanyl-arginy-l-arginyl-4-methoxy-beta-naphthylamide, a synthetic substrate highly specific for CB1 at a pH optimum of 6.2. Fluorescence of the reaction product, 4-methoxy-beta-naphthylamine, was determined at 420 nm, using an activation beam of 292 nm. A series of concentrations of the amine was analyzed concomitantly as standard. Activity of beta-glucuronidase (beta-D-glucuronide glucuronohydrolase; EC 3.2.1.31) was determined at pH 4.5 by the spectrophotometric method of Fishman as modified by Msa et al. (31), using the substrate phenolphthahlicin-beta-D-mono glucosiduronic acid.

Alkaline phosphatase (EC 3.1.3.1) activity was determined in the presence of 5.1 mM magnesium acetate at pH 9.6 by the fluorometric method of Campbell and Moss as described elsewhere (38). Activity of succinate dehydrogenase (EC 1.3.99.1) was assayed at pH 7.4 as described by Pennington (36). Lactate dehydrogenase (EC 1.1.1.27) activity was determined at pH 8.8 by the method of Clark and Yochim (11).

Preparation of Liposomes with or without Entrapped Leupeptin

Liposomes composed of sphingomyelin, cholesterol, and octadecylamine were prepared by the method of Magee et al. (27). The procedure for entrapment of leupeptin, a peptide aldehyde (acyctyl- and propionyl-L-leucyl-L-leucyl-L-arginal), in liposomes was described previously (38). Liposomes were stored in suspension at 0°-4°C. Before use in experiments with isolated cells, liposomes were washed by centrifugation at 60,000 g for 30 min and then resuspended in divalent cation-free Ringer's solution. The final cholesterol contents of vehicle control- and leupeptin-liposomes, as determined by the method of Albers and Lowry (2), were not significantly different and averaged 12.0 \pm 0.1 µg of cholesterol per 5 ml of medium in 30 separate experiments.
Analysis of Net Rate of Aggregation of Isolated Endometrial Cells

The degree of ceil-to-cell adhesion was determined by methods similar to those described previously (38). Isolated endometrial cells were filtered through 63-μm nylon mesh. Thereafter, they (ca. 2 × 10⁹ cells) were incubated in 5 ml of divalent cation-free Ringer’s solution with 0.1 mM EDTA and 1 mM sodium pyruvate. After 15, 30, or 60 min with shaking (60 oscillations/min) in the presence or absence of additives, cell suspensions were filtered with stirring through a stop-flow apparatus. The apparatus was similar to that described elsewhere (40), except that a 63-μm nylon mesh filter was fitted to the base (cf. reference 38). Cells retained by the nylon mesh were then washed in the apparatus for two successive 1-min periods with 5-ml volumes of the divalent cation-free Ringer’s solution. Cells not retained by the nylon mesh were sedimented by centrifugation at 400 g (5 min), sonically disrupted, and analyzed for DNA content. Cells remaining on the nylon filter by virtue of aggregation were also sonically disrupted, and samples were collected for determination of DNA. Viability of cells (see below) decreased by only 1% after 30-min incubation and filtration through the stop-flow apparatus.

Determination of Cell Viability and Test Reagents

Viability of cells was evaluated by a dye-exclusion method. Endometrial cells were suspended in Ringer’s solution with 0.05% nigrosin (NA 0683; Allied Chemical Corp., Specialty Chemicals Div., Morristown, N. J.) for 5-10 min at 37°C. In the present investigation, 96 ± 1% of cells sampled in each experiment excluded the vital dye. Preparations in which cell viability was <92% were discarded. The viable cells in the several experiments were incubated with or without estradiol-17α (E₂α), E₂β, or estriol (E₃; all from Schering Corp.), or with vehicle control (0.02% ethanol, final concentration). Proteinase inhibitors, including ovomucoid (type II-0), L-5-amino-1-(p-toluenesulfonyl)-amidopentyl-chloromethyl ketone (TLCK), iodoacetate, and soybean trypsin inhibitor (SBTI), were obtained from Sigma Chemical Co. (St. Louis, Mo.).

Data Analysis

Variability of the data is expressed as standard error of the mean with the number of independent experiments in parentheses. The t test was applied to assess the differences between the means of paired or independent data, as appropriate.

RESULTS

Effect of Estrogens on Con A Binding and Con A-Mediated Hemadsorption To Endometrial Cells

Previous studies have shown that endometrial cells possess specific binding-sites for F-Con A. Cells bind the lectin maximally at a concentration of 100 μg Con A/ml with 3-min incubation at 22°C (42). Accordingly, Con A binding was determined under these conditions after incubation of cells with or without estrogens (E₂β, E₃) for 30 min. Although the absolute levels of lectin bound were slightly elevated by treatment of endometrial cells with estrogens at 1 × 10⁻⁶ M, the hormone elicited no statistically significant increase in Con A binding as compared to levels determined in paired cells exposed only to vehicle control (Fig. 1).

Con A also mediates the adsorption or cross-linkage of homologous erythrocytes to epithelial cells labeled with the multivalent lectin (37, 38, 42). Therefore, the association of rat erythrocytes with endometrial cells exposed to 100 μg Con A/
ml (3 min, 22°C) was determined as described previously (42). These analyses provide a quantitative estimate of lectin-mediated cellular agglutinability, a property known to be expressed more markedly in transformed cells than in their normal counterparts (6, 9). Accordingly, hemadsorption to isolated endometrial cells was determined immediately after their incubation with estrogens. Treatment with $1 \times 10^{-9}$ M E2 elicited marked increments in Con A-mediated hemadsorption by endometrial cells to 237% of that by paired controls ($P < 0.001$; Fig. 1). A more moderate elevation in cellular agglutinability to 178% of control levels ($P < 0.01$) was found also among cells exposed to $1 \times 10^{-4}$ M E3 for 30 min.

Influence of Proteinase Inhibitors on Con A Binding and Con A-Mediated Hemadsorption to Endometrial Cells Treated with E2

The accentuated agglutinability of transformed cells has often been related to increased proteinase activity associated with growth stimulation (cf. reference 33). In consideration of these data, lectin-mediated hemadsorption by endometrial cells treated with $1 \times 10^{-9}$ M E2 for 30 min was determined after incubation of cells with or without proteinase inhibitors.

Cells were treated for 30 min with $5 \times 10^{-4}$ M TLCK, a chloromethyl ketone selective for certain serine and thiol proteinases (7, 35), or $4.5 \times 10^{-7}$ M leupeptin, a potent antagonist of trypsin and cathepsin B1 (59, 61). Such treatments did not significantly alter the level of Con A binding to cells as compared to paired vehicle controls ($P > 0.40$; Fig. 1). However, incubation of cells with either TLCK or leupeptin before treatment with E2 evoked a marked decline in the amount of Con A-mediated hemadsorption to the endometrial cells as compared with the response in the absence of the relevant inhibitor ($P < 0.001$; Fig. 1).

Ovomucoid is a trypsin inhibitor with weak antagonism toward cathepsin B1 (35, 59). Treatment of endometrial cells with this compound at relatively high concentrations elicited partial reductions in Con A-mediated hemadsorption, but not Con A binding, to cells subsequently exposed to E2, as compared with paired cells treated with E2 in the absence of ovomucoid ($P < 0.05$; Fig. 1). In contrast to the degree of effectiveness of leupeptin, TLCK, and ovomucoid, the serine proteinase inhibitor, SBTI, which has no influence on the activity of thiol proteinases such as cathepsin B1 (35), failed to curtail the enhanced Con A-mediated hemadsorption to endometrial cells attributable to E2 (Fig. 1). Therefore, only those agents which are effective suppressors of certain thiol and serine proteinases (e.g., TLCK, ovomucoid, leupeptin) were capable of countering the influence of E2 treatment on lectin-mediated hemadsorption to endometrial cells.

Iodoacetate suppresses the activity of cathepsin B1-like proteinases by reacting with the essential thiol group (7, 35, 59). Prior treatment of endometrial cells with $5 \times 10^{-6}$ M iodoacetate (30 min) elicited a significant reduction in Con A-mediated hemadsorption, but not Con A binding, to cells subsequently exposed to E2, as compared with paired cells treated with E2 in the absence of iodoacetate ($P < 0.001$; Fig. 1). Thus, these additional data strengthen the inference that surface alterations in response to E2 treatment are associated with enhanced thiol proteinase activity of the cathepsin B1-type.

Effect of Prior Treatment with Leupeptin

Endometrial cells were incubated with or without $1 \times 10^{-9}$ M estrogen for 5 min before exposure to 100 µg Con A/ml for 3 min at 22°C as described above. In light of the enhanced net rate of cellular aggregation after exposure to active estrogen (see below), a submaximal time period of 5 min of hormone treatment was used in the present series. This expedient permitted observations on the distribution of F-Con A at the surfaces of predominantly individual cells. F-Con A binding to cells was evaluated by dark-field ultraviolet fluorescence microscopy. When 500 µg unmodified Con A/ml was added with 100 µg F-Con A/ml, fluorescence at the cell surfaces was minimal, thus demonstrating the specificity of F-Con A binding (Fig. 2a). In the absence of excess unlabeled lectin, F-Con A was distributed in apparently random patches over the surfaces of the majority of cells treated with hormone vehicle (Fig. 2b) or E2 (Fig. 2c). In contrast, addition of E2 to cells evoked an apparent rearrangement of binding sites for Con A into polar clusters at the surfaces of ~25% of all isolated cells observed (Fig. 2d). Such “capping” was not observed in
cells exposed only to hormone vehicle or E₂α for up to 30 min. The remaining essentially 75% of E₂β-treated cells exhibited a nonpolar distribution of label similar to that shown in Fig. 2b. In additional experiments, endometrial cells were exposed to 4.5 × 10⁻⁷ M leupeptin for 30 min before 5-min incubation with hormone vehicle (Fig. 2e) or E₂β (Fig. 2f). Such prior treatment with proteinase inhibitor partially blocked the characteristic redistribution of binding sites for F-Con A observed in cells incubated only with E₂β (Fig. 2d).

Analyses of [³H]E₂β Binding by Intact Endometrial Cells and Cytosol Fractions and the Effects of Proteinase Inhibitors

As previously noted, the specific accumulation of E₂β by intact cells derived from endometrium is a saturable process, with maximal binding of hormone at 1 × 10⁻⁶ M after 30-min incubation at 22°C (44). To determine the effects of proteinase inhibitors on the extent of E₂β binding in such cells, specific accumulation of 1 × 10⁻⁶ M E₂β was measured after 30-min prior incubation with or without the various concentrations of leupeptin or SBTI indicated in Fig. 3. Treatment of the endometrial cells with 50–250 µg SBTI/ml did not influence the degree of specific E₂β accumulation relative to that of cells treated with vehicle alone (all at P > 0.80). Exposure of cells to 2 × 10⁻⁵ M leupeptin likewise elicited no significant alteration in the extent of estradiol binding as compared to controls (P > 0.90). In contrast, treatment with leupeptin at concentrations ranging from 9 × 10⁻⁶ to 2 × 10⁻⁴ M promoted progressive reduction in E₂β uptake to ~60% of control levels at the latter concentration. Testing of E₂β binding by intact cells in the presence of higher levels of leupeptin was not possible because exposure of cells to 10⁻³ to 10⁻⁴ M concentrations of leupeptin was found to reduce the percent of viable cells below acceptable levels (cf. reference 61).
FIGURE 3 Effects of proteinase inhibitors on \[\text{[H]}\text{E}_2\beta\] binding to intact endometrial cells and to high-speed supernatant fractions obtained after cell homogenization. Intact cells were incubated in Ringer’s solution with (closed circles) or without leupeptin or SBTI (open circles) at the concentrations indicated for 30 min before exposure to $1 \times 10^{-8}$ M \[\text{[H]}\text{E}_2\beta\] for 30 min at 22°C. Cytosol fractions in Tris-buffered solution were incubated with (triangles) or without leupeptin for 1 h at 4°C before incubation with hormone for 2 h at 4°C. Only specific binding (defined as the difference in bound steroid between paired tubes, one of which contained a 200-fold excess of unlabeled E$_2$B throughout the experiment) is shown. All values are given as percent of specific E$_2$B binding in paired cells incubated without proteinase inhibitors (i.e., 24,597 ± 824 dpm [H]E$_2$B bound/mg cell protein) or paired supernatant fractions not exposed to leupeptin (i.e., 31,730 ± 1050 dpm [H]E$_2$B bound/mg cytosol protein) in two to three independent experiments. Means of duplicate determinations are shown without the SEM given in the cases of triplicates.

The influence of leupeptin on binding of \[\text{[H]}\text{E}_2\beta\] by endometrial cells at 22°C was analyzed further in equilibrium-binding experiments (Fig. 4). After a 30-min incubation with or without $4.5 \times 10^{-7}$ M leupeptin, cells ($2 \times 10^4$/ml) were exposed to a series of \[\text{[H]}\text{E}_2\beta\] concentrations ranging from $1 \times 10^{-10}$ to $2.5 \times 10^{-8}$ M for 30 min. The specific binding of E$_2$B by cells treated with or without the peptide aldehyde is presented in Fig. 4. To obtain both the concentration of specific binding sites for E$_2$B and the equilibrium constant for the binding reaction in each group, data in Fig. 4 were analyzed further (see Fig. 4, inset) by the notation of Scatchard (49). Mathematical resolution of the binding data by the method of least squares yields an apparent molar dissociation constant for the binding process of $1.9 \times 10^{-9}$ among cells not exposed to leupeptin, as compared to $2.0 \times 10^{-8}$ in cells pretreated with the proteinase inhibitor. The total number of E$_2$B binding sites per cell at saturation corresponds to 21,110 in control cells and to 20,840 in leupeptin-treated cells (see Fig. 4, inset). Thus, prior exposure of endometrial cells to leupeptin elicits a reduction in the affinity of hormone binding by intact cells without substantially affecting the apparent number of binding sites per cell. Specific binding of $1 \times 10^{-9}$ M \[\text{[H]}\text{E}_2\beta\] to
macromolecular components of cytosol from endometrial cells was determined after 2-h incubation at 4°C. Treatment of the supernatant fractions with $2 \times 10^{-6}$ to $2 \times 10^{-8}$ M leupeptin at 4°C for 1 h before incubation with hormone elicited no significant alteration in $E_2\beta$ binding as compared to control levels ($P > 0.70$; Fig. 3). In additional studies, specific binding of $2 \times 10^{-10}$ or $6 \times 10^{-10}$ M $[^{3}H]E_2\beta$ to macromolecular components of cytosol was likewise uninfluenced by prior incubation with $4.5 \times 10^{-7}$ M leupeptin (not shown). Thus, only binding of $E_2\beta$ by intact endometrial cells is reduced by treatment with the peptide aldehyde.

**Activities of Hydrolytic Enzymes Released into the Particle-Free Medium from Endometrial Cells and the Effect of Estrogen Treatment**

Considerable biochemical and morphologic evidence demonstrates that estrogens elicit rapid reduction in the structural latency of lysosomal components in specific target cells (cf. reference 57). Determinations of the extracellular release of a variety of hydrolases were undertaken among endometrial cells treated for 30 min with several estrogenic congeners. The results are presented in Table I. $E_2\beta$, but not $E_2\alpha$, elicited a statistically significant increase in the extracellular release of the acid hydrolases cathepsin B1 and $\beta$-glucuronidase from cells incubated with steroid at a concentration of $1 \times 10^{-8}$ M, as compared with paired cells exposed to control vehicle during a 30-min incubation period. A moderate elevation of extracellular cathepsin activity was also detected among endometrial cells treated with $1 \times 10^{-9}$ M of the less active estrogen, $E_2$, relative to that at the external surfaces of paired controls. In contrast, treatment with estrogens did not significantly enhance the extracellular activities of several nonlysosomal enzymes including alkaline phosphatase (Table I), nor of succinate dehydrogenase and lactate dehydrogenase (not shown). Activities of the latter two dehydrogenases were not detectable in extracellular media from either control or estrogen-treated cells. Thus, endometrial cells treated with physiologically potent estrogens appear to release selectively lysosomal enzymes at the external cell surface. The more sensitive and consistent indicator of treatment with estrogens by this criterion was the thiol proteinase, cathepsin B1.

**Inhibition by Liposome-Entrapped Leupeptin of Estrogen-Induced Incorporation of $[^{3}H]$Thymidine into Macromolecular Form and of the Proliferative Response to Hormone**

Mounting evidence indicates that an endogenous proteinase may contribute to the estrogenic regulation of cell proliferation in uterine tissues (24, 47, 59). The data presented above likewise suggest that a cellular proteinase sensitive to marked inhibition by leupeptin is involved in the mediation of specific surface alterations induced by $E_2\beta$ treatment. These data prompted us to analyze the mitogenic actions of estradiol in primary cultures of endometrial cells, with and without modification of leupeptin-sensitive proteinase activity.

Endometrial cells cultured in CDM showed no significant growth response to treatment with $1 \times 10^{-9}$ M $E_2\alpha$. Thus, $[^{3}H]$thymidine incorporation, determined after 20-h ($P > 0.40$), and cell proliferation after 24-h ($P > 0.90$; data not shown) or 48-h ($P > 0.20$) exposure to this relatively inert estrogen were essentially unchanged from control levels (Fig. 5a). In contrast, after treatment with $1 \times 10^{-8}$ M $E_2\beta$, $[^{3}H]$thymidine incorporation was enhanced to $241 \pm 6\%$ of controls ($P < 0.001$), while gross cell numbers after 24 and 48 h increased to $138 \pm 2\%$ ($P < 0.01$; data not shown) and $208 \pm 5\%$ ($P < 0.001$), respectively, of control levels.

The effect of leupeptin on $E_2\beta$-induced alterations in cell growth was first tested by an initial incubation of cell cultures for 30 min with $7 \times 10^{-8}$ M of the proteinase inhibitor. The supernatant medium was then removed by centrifugation at 400 g for 5 min. The sedimented cells were washed once by centrifugation, resuspended in culture medium, and then divided into two portions. One portion was analyzed for total cellular cathepsin B1 and additional hydrolase activities (see Table II), while the other was incubated further in the presence of $E_2\beta$ or control vehicle. Such treatment with leupeptin reduced total cellular cathepsin B1 activity to $77 \pm 5\%$ of control levels, without demonstrable effect on $\beta$-glucuronidase, alkaline phosphatase, or succinate dehydrogenase activities (Table II). In the second portion of the leupeptin-treated cells, subsequent incubation with $E_2\beta$ resulted in an increase in $[^{3}H]$thymidine incorporation to 215
TABLE 1

Effect of Estrogen Treatment for 30 min on Activities of Hydrolytic Enzymes in the Particle-Free Media from Suspensions of Endometrial Cells

| Group                  | Extracellular enzyme activity | pmol/min/mg cell protein * |
|------------------------|-------------------------------|---------------------------|
| Control (0.02% ethanol)| Cathepsin B                  | 43 ± 3 (5)                |
|                        | β-Glucuronidase               | 2,227 ± 30 (5)            |
|                        | Alkaline phosphatase          | 10 ± 1 (5)                |
| E₂α (1 x 10⁻⁶ M)       |                               | 45 ± 1 (3)                |
|                        |                               | 2,272 ± 42 (3)            |
| E₂β (1 x 10⁻⁶ M)       |                               | 88 ± 1 (5)                |
|                        |                               | 2,606 ± 48 (5)            |
| E₃ (1 x 10⁻⁶ M)        |                               | 11 ± 1 (5)                |

* After 30-min incubation at 22°C with or without estrogens, enzyme activities detected in particle-free supernatant fractions from cell suspensions were <0.5% of total available cell hydrolase activities; no significant change in total enzyme contents was found after 30-min E₂β treatment (P > 0.80 vs. controls).

† Value significantly different from control at P < 0.001.

§ Value significantly different from control at P < 0.05.

|| Value significantly different from control at P < 0.01.

Figure 5 The influence of free or liposome-entrapped leupeptin on estrogen-enhanced incorporation of [³H]thymidine into acid-precipitable form and on proliferation of endometrial cells. Values of [³H]thymidine incorporation after 20 h (clear bars) and cell numbers after 2 d of incubation (shaded bars) were obtained in three to four independent experiments in the presence and absence of 1 x 10⁻⁸ M estrogen, as indicated. See text for details.

± 18% of appropriate controls not exposed to hormone (P < 0.001; Fig. 5b). An increase in cell numbers limited to 150% of controls (P < 0.001) was also found in cultures that had been treated with free leupeptin and then incubated in the presence of E₂β for 2 d (Fig. 5b). These combined data reflect a moderate reduction in cellular sensitivity to E₂β resulting from prior treatment with leupeptin as compared to these criteria of hormonal response in the absence of the proteinase inhibitor (Fig. 5a).

To achieve more efficient delivery of leupeptin to the cell interior, the proteinase inhibitor was entrapped in cationic liposomes. Cell cultures were incubated with liposomes containing leupeptin at a final concentration of 7 x 10⁻⁸ M. In preliminary experiments, prolonged exposure of cells to liposomes as such was found to reduce the percent of viable cells below acceptable levels. Therefore, the incubation medium was removed after 30 min and replaced with fresh medium before proceeding to assessment of estrogen-in-
TABLE II

Effects of Brief Incubation of Endometrial Cells with Free or Liposome-Entrapped Leupeptin on Total Activities of Various Hydrolytic Enzymes

| Treatment                  | Cathepsin B1 (nmol/min/mg cell protein) | β-Glucuronidase (nmol/min/mg cell protein) | Alkaline phosphatase (nkat/min/mg cell protein) | Succinate dehydrogenase (nmol/min/mg cell protein) |
|---------------------------|----------------------------------------|------------------------------------------|-----------------------------------------------|--------------------------------------------------|
| Control vehicle           | 14.2 ± 2.0 (3)                         | 3.040 ± 120 (3)                          | 4.7 ± 4 (3)                                   | 7.9 (2)                                          |
| Leupeptin (7 × 10^{-8} M) | 10.9 ± 0.7 (3)                         | 3.201 ± 112 (3)                          | 5.0 ± 3 (3)                                   | 7.2 (2)                                          |
| Control-liposomes         | 13.1 ± 1.5 (3)                         | 2.800 ± 140 (3)                          | 4.3 ± 3 (3)                                   | 6.8 (2)                                          |
| Leupeptin-liposomes       | 4.6 ± 0.8 (3)                          | 2.982 ± 121 (3)                          | 4.5 ± 4 (3)                                   | 7.0 (2)                                          |

* Endometrial cells were treated for 30 min as indicated in the table and then sedimented and washed once with centrifugation at 400 g for 10 min. Sedimented cells were sonicated and then solubilized in 0.1% (vol/vol) Triton X-100 at 4°C (2 h). Portions of the sediment thus solubilized were analyzed for protein and enzyme activities as described in the text.

‡ Value significantly different from paired control at P < 0.05.
§ Value significantly different from paired control at P < 0.001.

TABLE III

Effect of Estrogens on the Aggregation of Endometrial Cells and the Influence of Prior Treatment with Leupeptin

| Group                                      | µg DNA cells retained/µg DNA input cells |
|--------------------------------------------|-----------------------------------------|
| Control (0.02% ethanol)                    | 0.00 ± 0.00 (4)                         |
| E2α (1 × 10^{-8} M)                        | 0.00 (2)                                |
| E2β (1 × 10^{-9} M)                        | 0.34 ± 0.02 (4)                         |
| Leupeptin (4.5 × 10^{-7} M) and vehicle control | 0.00 (2)                                |
| Leupeptin and E2β                          | 0.03 ± 0.01 (3)                         |

* Endometrial cells were suspended in divalent cation-free Ringer’s with 0.1 mM EDTA and 1 mM sodium pyruvate, and filtered over 63-µm nylon mesh. Filtered cells were then incubated for 30 min at 22°C with or without estrogens as indicated in the table. In experiments with leupeptin, cells were incubated with the peptide aldehyde for 30 min before the 30-min treatment with E2 or hormone vehicle. At the end of the treatment period, cells were filtered again over 63-µm nylon and washed twice with 2 vol of medium. Cells retained by the filter and those passed through the 63-µm mesh were collected and analyzed for DNA. The extent of intercellular adhesion was then expressed as the ratio of µg DNA of cells retained by the filter to µg DNA of all cells in suspension at the start of the procedure (i.e., input cells).

‡ Value significantly different from that of paired control at P < 0.001.

Effects of Estrogens on Aggregation of Endometrial Cells and the Influence of Prior Treatment With Leupeptin

Recent studies demonstrate that chemical transformation of epithelial cells from urinary bladder is associated with an increase in the rate of intercellular adhesion (38). The net rate of aggregation of endometrial cells during 15-, 30-, and 60-min incubation was analyzed under similar conditions, as specified in Table III. The net rate as measured here is a function of both the rate of formation and the rate of dissociation of intercellular adhesions.

Essentially no aggregation of endometrial cells
was found after 15-, 30-, or 60-min incubation in the presence of hormone vehicle or $1 \times 10^{-9}$ M E$_2\alpha$. In contrast, exposure of cells to $1 \times 10^{-9}$ M E$_2\beta$ elicited aggregation of 16 ± 2% ($n = 3$) of input cells by 15 min, 34 ± 2% by 30 min (see Table III), and 35% ($n = 2$) by 60 min. Thus, indications of apparently maximal intercellular adhesion were evident by 30 min incubation time, and these values are shown in Table III.

Prior exposure of cells to $4.5 \times 10^{-7}$ M leupeptin for 30 min did not elicit aggregation of endometrial cells during a subsequent 30-min incubation with hormone vehicle (Table III). However, cells exposed to hormone for 30 min after prior treatment for 30 min with leupeptin exhibited only a minimal level of intercellular adhesion which was significantly less than that of cells incubated with E$_2\beta$ in the absence of the proteinase inhibitor ($P < 0.001$; Table III).

**Fractionation of Endometrial Cells with Differential Responsiveness to E$_2\beta$**

The growth, lectin-mediated hemadsorption, and proteinase-secretory properties of cells fractionated by virtue of their differential adhesiveness were analyzed in further experiments. After treatment of cells with $1 \times 10^{-9}$ M estrogen or vehicle control for 30 min, adherent cells retained in the stop-flow apparatus were collected and maintained apart from nonadherent cells that were not excluded by the 63-μm filter.

| Group       | Prior treatment | Extracellular release of cathepsin B1 activity in 30 min (% total cellular activity) | Con A-mediated hemadsorption (absorbance, 418 nm/mg endometrial cell protein) |
|-------------|-----------------|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| Nonadherent | Control (0.02% ethanol) | 0.3 ± 0.0 (3)                                                                         | 0.24 ± 0.02 (3)                                                                 |
|             | E$_2\beta$ ($1 \times 10^{-9}$ M) | 0.4 ± 0.1 (3)                                                                         | 0.31 ± 0.02 (3)                                                                 |
| Adherent    | E$_2\beta$ ($1 \times 10^{-9}$ M) | 1.0 ± 0.1§ (3)                                                                         | 0.93 ± 0.08 (3)§                                                                |

* Endometrial cells were incubated with $1 \times 10^{-9}$ M E$_2\beta$ or hormone vehicle for 30 min and then filtered over 63-μm nylon mesh as described in the text. Cells retained by the filter (adherent cells) and those passed through the mesh (nonadherent cells) were collected, sedimented by centrifugation at 400 g (5 min), and resuspended in complete Ringer's solution. As in experiments shown in Table III, no adherent cells resulted from exposure to hormone vehicle. For studies of extracellular cathepsin B1 release, cells (approx. $3 \times 10^9$/ml) were incubated for 30 min at 22°C and then prepared for enzyme analyses as described in the text. The total cellular activity of cathepsin per mg cell protein was not significantly different among the several treatment groups. For studies of Con A-mediated hemadsorption to endometrial cells, approx. $2 \times 10^9$ cells per 5 ml of Ringer's solution were incubated for 3 min with 100 μg Con A/ml and then prepared for assessment of hemadsorption to the lectin-labeled cells as described in the text.

§ Values significantly different from that of paired control at $P < 0.01$.

§§ Value significantly different from that of paired control at $P < 0.05$. 

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After isolation of these fundamentally dissimilar fractions of the original mixed cell population, they were incubated for 30 min and analyses for cathepsin B1 activity in the extracellular media were conducted (Table IV). Proteinase activity released by adherent cells was elevated to 250 ± 25% of that by nonadherent cells that had been exposed to E$_2\beta$ while still in suspension with their adherent counterparts ($P < 0.05$). Moreover, the adherent cells exhibited levels of cathepsin B1 secretion more than 3.3-fold greater than that of cells exposed only to control vehicle.

The association of erythrocytes with adherent and nonadherent endometrial cells exposed to Con A was also determined immediately after cell fractionation. As shown in Table IV, adherent as well as nonadherent cells, both of which had been exposed to E$_2\beta$, exhibited increases in Con A-mediated hemadsorption as compared to those levels in vehicle-control cells. However, the extent of lectin-mediated hemadsorption to the adherent group of hormone-treated cells exceeded that of nonadherent cells exposed to E$_2\beta$ by three times ($P < 0.01$). This parallel between the results from two completely independent probes for E$_2\beta$-induced changes in membrane properties is striking. No significant difference in Con A binding to endometrial cells was elicited by treatment with E$_2\beta$ as compared to the binding levels measured in cells treated only with hormone vehicle (both at $P > 0.50$; data not shown).
Incorporation of [3H]thymidine in cell fractions cultured in CDM for 22 h after isolation by the adhesion procedure was also determined (Table V). Thymidine incorporation by adherent cells treated with E2β was 528 ± 26% of that by nonadherent cells exposed to hormone (P < 0.001), and 569 ± 28% of that by cells in the vehicle-control group (Table V). These marked increments in nucleotide incorporation were consistent with independent measurements of increased DNA contents in paired cell cultures incubated under the same conditions for 26 and 50 h (Table V). Thus, isolation of a fraction of endometrial cells by selection for an E2β-induced membrane alteration also yields a cell fraction highly sensitive to the mitogenic effects of the hormone.

**DISCUSSION**

The present studies provide evidence that early alterations induced by E2β at the surfaces of endometrial cells, as well as enhanced growth evoked by the hormone in primary culture, are both associated with increased availability of an endogenous leupeptin-sensitive proteinase. Treatment of cells with E2β evokes a sharp rise in the access of cathepsin B1-like activity to the external cell surfaces. In contrast, extracellular activity of the proteinase is substantially less in cells exposed to E3, a weak mitogen (cf. reference 4), and unchanged from that of controls in cells treated with the relatively inert congener, E2α. Additional investigations also show a marked enhancement of cathepsin B1 activity in the extracellular environment of isolated cells from endometrium and urinary bladder treated with either diethylstilbestrol or a carcinogenic nitrosamine, respectively (38, 43). Moreover, other reports demonstrate a striking immunohistochemical localization of cathepsin B1 at the surfaces of transformed and tumor cells (55) and marked elevation of cathepsin B1-like activity in the sera of patients with cancer (46).

Recent data indicate that reorganization of the cell surface may be an obligatory step in the normal progression of cells to mitosis (9, 10, 14, 33, 42, 53). In this context, cathepsin-mediated alterations in the enzymatic activity or in the integrity of membrane-associated proteins by limited proteolysis may contribute to such changes in the composition or distribution of membrane components (cf. reference 51). The present data provide evidence that estrogen-induced secretion of a cathepsin B1-like proteinase may promote the expression of cell surface modifications well before initiation of DNA synthesis due to hormone. Treatment of cells with E2β, but not E2α, for only 5 min elicited in ~25% of exposed cells redistribution into polar clusters of Con A-binding sites normally disposed in random patches at the external cell surface. Due to the submaximal period of hormone exposure in the latter experiment, the reported percentage is probably a minimum estimate of the actual proportion of responsive cells. However, the results establish that E2β-induced redistribution of lectin-binding sites occurs at target cell surfaces either before or concomitantly with a hormone-stimulated increase in Con A-

**Table V**

| Group          | Prior treatment | dpm [3H]thymidine incorporated/μg DNA/22 h | μg DNA after 26 h/initial μg DNA | μg DNA after 50 h/initial μg DNA |
|---------------|-----------------|------------------------------------------|---------------------------------|---------------------------------|
| Nonadherent cells | Control (0.02% ethanol) | 118 ± 4 (4) | 1.02 ± 0.01 (3) | 1.06 ± 0.05 (3) |
|                | E2β (1 x 10^-9 M) | 127 ± 5 (4) | 1.06 ± 0.04 (3) | 1.09 ± 0.08 (3) |
| Adherent Cells | E2β (1 x 10^-9 M) | 671 ± 33 (4) | 1.96 ± 0.06 (3) | 3.86 ± 0.07 (3) |

*Endometrial cells were filtered over 63-μm nylon mesh after treatment with or without 1 x 10^-9 M E2β for 30 min at 22°C. As indicated in Table III, ~34% of cells exposed to E2β were retained by the filter (adherent cells), while the remainder were not (nonadherent group); vehicle-control cells were not retained by the filter. Groups of adherent and nonadherent cells were sedimented by centrifugation at 400 g for 5 min. Cells were then suspended in CDM and allowed to attach to culture dishes at 37°C in a humidified atmosphere of 5% CO2 in air. After 2 h, the medium was exchanged for 5 ml of fresh CDM. Determinations of DNA synthesis among the several cell fractions were then begun with approx. 5 x 10⁵ cells/dish in each experiment. Incorporation of [3H]thymidine into acid-precipitable form was analyzed 22 h after fractionation of cells by the adhesion procedure. In independent experiments, levels of total cell DNA were determined after 26 or 50 h.

‡ Value significantly different from that of paired control at P < 0.001.
mediated hemadsorption to endometrial cells. The minimal increases in the levels of lectin binding by E\textsubscript{2\beta}-treated as compared with control cells in the present and in previous studies (42, 43) are inadequate to explain the marked differences in Con A-mediated hemadsorption to endometrial cells. Other investigators have reported a clustering of lectin-binding sites and an increase in lectin-mediated agglutination in transformed cells of various types and have considered both events to be a consequence of increased lateral movement of lectin-binding components of the plasma membrane (cf. references 9 and 33). The contribution to lectin reactivity of the latter and additional factors such as E\textsubscript{2\beta}-induced alterations in microvillous formations at the surfaces of endometrial cells (cf. references 5 and 33) remains to be evaluated.

Additional evidence of plasma membrane alterations early after E\textsubscript{2\beta} treatment was found in studies of intercellular adhesion. The active estrogen, but not E\textsubscript{2\alpha}, elicited marked aggregation of ~34% of endometrial cells. Such E\textsubscript{2\beta}-induced changes were sensitive to blockade by prior treatment with leupeptin. Moreover, selection of highly adherent cells by the adhesion method after estrogen treatment yielded a cell fraction that was profoundly effective without further challenge with the hormone in the release of cathepsin B1, Con A-mediated hemagglutination, and DNA synthesis as compared to their nonadherent counterparts. These results are consistent with previous work which indicated that ~40% of similarly isolated endometrial cells bear surface-binding sites specific for interaction with immobilized estradiol (44). In independent studies in which endometrial cells were isolated from uteri of ovariectomized rodents by comparable procedures (1, 54), the authors estimated that 75–85% of the cell population was composed of luminal and glandular epithelial cells, with the remainder, cells of the connective tissue stroma. Autoradiographic studies of [\textsuperscript{3}H]thymidine incorporation in thin uterine sections show that 70–80% of luminal epithelial cells, 20–30% of glandular epithelial cells, and essentially no stromal cells are labeled with the nucleotide after 15-h treatment with E\textsubscript{2\beta} in vivo (28). Thus, the present results obtained by in vitro treatment with hormone are consonant with the latter in vivo observation that the response of endometrial cells to E\textsubscript{2\beta} is not uniform among the several cell types. Additional work is necessary to establish the nature and properties of such preferentially responsive cells. The present methods of cell fractionation by surface-membrane behavior should aid in such studies on the disposition of functionally divergent cells in the uterus.

Lysosomes of responsive cells are known to concentrate a wide variety of chemical mitogens (cf. reference 3) and possess binding sites with specificity for E\textsubscript{2\beta} (16, 21, 57). Further, the redistribution of limited concentrations of lysosomal hydrolases (59) and antigens from this organelle (58) to the nuclear compartment and external surfaces of target cells after E\textsubscript{2\beta} exposure has also been reported. These cellular responses to hormone may correspond to the coupled processes of endocytosis and lysosomal fusion (cf. references 12 and 57), processes which are being exploited as a means of introduction of antitumor drugs into the cell interior (13). Similarly, the present data show that treatment of endometrial cells with leupeptin-loaded liposomes evokes a marked suppression of the anticipated growth response after E\textsubscript{2\beta} exposure. Such observations lend further support to the concept that a leupeptin-sensitive proteinase may promote enhanced genic expression induced by estrogens (24, 57, 59). Independent studies show that subcutaneous administration of milligram amounts of leupeptin to normal cyclic rats elicits a dramatic decrease in uterine weight and DNA content, and in fertility rates (24). Moreover, leupeptin also inhibits the stimulation of [\textsuperscript{3}H]thymidine incorporation by phytohemagglutinin in lymphocytes (48), restricts the growth of normal and polyoma-transformed kidney cells in culture (29), and diminishes tumorigenesis induced by a variety of chemicals (38, 61).

The inhibition of endometrial cell responses to E\textsubscript{2\beta} by leupeptin may also be attributable, in part, to reduction of hormone accumulation by intact cells after exposure to the proteinase inhibitor as shown in the present experiments. Independent studies have demonstrated that various sulfhydryl-group blocking reagents, including iodoacetate and iodoacetamide, potential inhibitors of cathepsin B1 activity (7, 35), restrict the entry of estrogen into uterine cells while exerting markedly less inhibition of the binding of the steroid by cytosol receptor (30, 60). Iodoacetamide was also found to suppress the nuclear accumulation of estradiol-receptor complexes (30). It is conceivable that both leupeptin and sulfhydryl-group blockers are acting on a proposed Ca\textsuperscript{2+}-activated enzyme (47, 52, 59) that may be necessary for the estrogen-
triggered transformation (23) of the native estradiol-receptor complex into a modified (52) molecule capable of binding to nuclear acceptor sites. A crude preparation of an intracellular Ca2+-dependent proteinase that exhibits maximal activity at pH 8.5 and that possesses high affinity for native estradiol-receptor from calf uterus has recently been described (47). Limited amounts of Ca2+-activated lysosomal proteinase, exhibiting a pH optimum of 6.2 with synthetic substrate and possessing additional cathepsin B1-like properties, are transferred rapidly into the nuclear compartment of target cells after E2β treatment (59). These considerations, as well as additional evidence for the occurrence of neutral proteinase activities with (24, 50) or without (34) a requirement for Ca2+ in estrogen-responsive tissues, require further investigation.

There are additional implications of the present data in light of recent evidence that binding sites with high affinity and specificity for E2β occur at the plasma membranes of responsive cells (38, 44) and may mediate the uptake of hormone (30, 57). Previous reports indicated that >70% of native estradiol-receptor components possess high affinity for target cell membranes and are sequestered in discrete extranuclear compartments before interaction with E2β (22). Thus, it is conceivable that enhanced availability of a leupeptin-sensitive proteinase at the surface membrane of target cells after E2β treatment may promote cleavage of an estrogen-binding fragment with high nuclear affinity from a membrane-localized binding component. The control of zymogen activation by limited proteolysis (cf. reference 32) offers ample precedent for this suggestion. Clearly, the concept of a proteinase-sensitive receptor component as presently postulated requires intensive analysis in future investigations. Such studies may, in turn, advance our understanding of the relation of membrane alterations elicited by estrogen to processes leading to mitogenesis in responsive cells.

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REFERENCES

1. ALBURGA, A., and E.-E. BAUMANN. 1968. Binding of estrogen in castrated rat endometrium in vivo and in vitro. Mol. Pharmacol. 4: 311-323.
2. ALBRECHT, W., W. CONWAY, and O. H. LOWRY. 1955. Fluorometric determination of 0.1 to 10 micrograms of cholesteryl. Anal. Chem. 27:1829-1831.
3. ANDERSON, A. C. 1974. Lysosomes in cancer cells. J. Clin. Pathol. 27: 43-50.
4. ANDERSON, J. N., E. J. PECK, JR., and J. H. CLARK. 1975. Estrogen-induced uterine responses and growth: Relationship to receptor estrogen binding by uterine nuclei. Endocrinology. 96:160-167.
5. ANDERSON, W. A., Y.-H. KANG, and E. R. DE SOMMER. 1973. Estrogen and progesterone-induced changes in endometrial topography of immature and cycling rats. J. Cell Biol. 64:69-703.
6. AUB, J. C., C. TERBAU, and A. LANCHESTER. 1963. Reactions of normal and tumor cell surfaces to enzymes. I. Wheat-germ lipase and associated mucopolysaccharides. Proc. Natl. Acad. Sci. U. S. A. 50: 613-619.
7. BARNETT, A. J. 1977. Cathepsin B and other thiol proteinases. In Proteinases in Mammalian Cells and Tissues. A. J. Barnett, editor. North-Holland Publishing Co., New York. 181-208.
8. BELLER, R. E., and B. W. O'MALLEY. 1976. The biology and mechanism of steroid hormone receptor interaction with the eukaryotic nucleus. Biochem. Pharmacol. 25:1-12.
9. BURGER, M. M. 1973. Surface changes in transformed cells detected by lectins. Fed. Proc. 32:91-101.
10. CARROT, D. H., and D. D. CUNNINGHAM. 1977. Initiation of chick cell division by trypsin action at the cell surface. Nature (Lond.) 268: 602-606.
11. CLARK, S. W., and J. M. YOCHEM. 1971. Effect of ovarian steroids on lactic dehydrogenase activity in endometrium and myometrium of the rat uterus. Endocrinology. 89:358-365.
12. CONN, Z. A. 1975. Macrophage physiology. Fed. Proc. 34:1725-1729.
13. DE DUVE, C., T. DE B. BERNESE, B. POOLE, A. TAOU, P. TULKENS, and F. VAN HOOF. 1974. Lysosomotropic agents. Biochem. Pharmacol. 23: 2495-2531.
14. DE TERRA, N. 1974. Cortical control of cell division. Science (Wash. D. C.) 184:530-537.
15. DEVLAMINGS, O. I. 1971. Effects of hormones on the cell cycle. In The Cell Cycle and Cancer. R. Berek, editor. M. Dekker, Inc., New York. 145-190.
16. FREIDAN, M. W., V. ORIGI, and S. L. HISA. 1977. Estrogene-binding protein from rat preputial gland. J. Biol. Chem. 252:3324-3327.
17. GERSCHONEN, L. E., and A. BURTON. 1976. Further studies on the regulation of cultured rabbit endometrial cells by diethylstilbestrol and progesterone. J. Steroid Biochem. 7:159-165.
18. GORLIN, J., and F. GOVERN. 1976. Current models of steroid hormone action: A critique. Arznei. Rev. Prakt. 58:625-630.
19. GORLIN, J., D. TUTT, G. SHINAGAL, D. SMITH, and A. NOTTRE. 1968. Hormone receptors: Studies on the interaction of estrogen with the uterus. Recent Prog. Horm. Res. 24:45-80.
20. HILL, B., and S. WHALLEY. 1975. A simple, rapid microassay for DNA. FEBS (Fed. Eur. Biochem. Soc.) Lett. 66:269-273.
21. HIBBS, P. C., and C. M. SEIDRO. 1974. Estradiol receptor functions of soluble proteins from target-specific lysosomes. J. Steroid Biochem. 5: 533-542.
22. JACKSON, V., and R. CHALKLEY. 1974. The cytoplasmic estradiol receptor of bovine uterus. J. Biol. Chem. 249:1627-1636.
23. JENSEN, E. V., T. SUZUKI, T. KAWamura, W. E. STRONG, P. W. JUNGblut, and E. R. DESOMMER. 1968. A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Natl. Acad. Sci. U. S. A. 60:623-638.
24. KATZ, J., W. TROLL, S. W. ADLER, and M. LEVINE. 1977. Antipeptide and leupeptin restrict estrogen-DNA synthesis and function in mice. Proc. Natl. Acad. Sci. U. S. A. 74:3754-3757.
25. KING, R. J. B., and W. I. P. MAJWANIA. 1974. Steroid-Cell Interactions. University Park Press, Baltimore.
26. Kornsman, S. G. 1968. Radio-ligand binding assay of specific estrogen using a soluble uterine macromolecule. J. Clin. Endocrinol. Metab. 28(1):151-154.

27. Magee, W. E., C. W. Goody, J. Schoenlicht, M. D. Smith, and K. Cherian. 1974. The interaction of cationic liposomes containing estradiol-17β-tetradecylphosphatidylethanolamine with cells in culture. J. Cell Biol. 63: 492-504.

28. Martin, L. C., and C. A. Frink. 1971. Oestrogen-estrogen interactions on miosis in target tissues. In Basic Actions of Sex Steroids on Target Organs. P. O. Hühn, F. Lenz, and P. Galand, editors. S. Karger, New York. 172-197.

29. McGreavy, A., and B. L. M. Hogan. 1974. Effect of inhibitors of proteolytic enzymes on the growth of normal and polyoma transformed BHK cells. Biochem. Biophys. Res. Commun. 60:348-354.

30. Melchion, E., M. Atger, and E.-E. Baulieu. 1973. Studies on oestrogen entry into uterine cells and on estradiol-receptor complex association with the nucleus--Is the entry of estrogen into uterine cells a protein-mediated process? Biochem. Biophys. Acta. 232:267-283.

31. Mura, B. U., R. P. Dore, and U. S. Sjö. 1965. Purification and properties of human liver β-galactosidase. J. Biol. Chem. 240:2811-2816.

32. Neubaitt, H., and K. A. Waleh. 1976. Role of proteolytic enzymes in biological regulation (A Review). Proc. Nas. Acad. Sci. U. S. A. 73: 3825-3832.

33. Nichol, G. L. 1976. Trans-membrane control of the receptors on normal and tumor cells. II. Surface changes associated with transformation and malignancy. Biochim. Biophys. Acta. 428:1-72.

34. Nordes, A. C., D. E. Hamilton, and J. H. Rudolph. 1973. The action of a human uterine proteinase on the estrogen receptor. Endocrinology. 90:210-216.

35. Otto, K. 1971. Cathepsins B1 and B2. In Tissue Proteases. A. J. Barrett and J. T. Dingel, editors. North-Holland Publishing Co., Amsterdam. 1-28.

36. Pennington, R. J. 1961. Biochemistry of dystrophic muscle. Mixochondrial succinate-tetrazolium reductase and adenosine triphosphatase. Biochem. J. 80:649-654.

37. Pietras, R. J. 1976. Vasopressin-induced redistribution of binding sites for concanavalin A on the surface of epithelial cells from urinary bladder. Nature (Lond.). 264:774-776.

38. Pietras, R. J. 1976. Heritable membrane alterations and growth associated with enhanced IgE-epitope-sensitive proteinase activity in epithelial cells exposed to dibutylintrinosamine in vitro. Cancer Res. 36: 1019-1030.

39. Pietras, R. J., T. W. Hutchins, and C. M. Szego. 1978. Hepatocyte plasma membrane subfractions enriched in high-affinity, low-capacity binding sites specific for estradiol-17β. Endocrinology. 102(Suppl.):76.

40. Pietras, R. J., and C. M. Szego. 1975. Steroid hormone-responsive, isolated endometrial cells. Endocrinology. 96:946-954.

41. Pietras, R. J., and C. M. Szego. 1975. Cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

42. Pieterse, J., R. E. Seiler, and C. M. Smith. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

43. Pieterse, J., R. E. Seiler, and C. M. Smith. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

44. Pieterse, J., R. E. Seiler, and C. M. Smith. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

45. Pieterse, J. R., and C. M. Szego. 1977. Blockade of estrogen uptake and endometrial cell responses to hormones by colchicine and protease inhibitors. Endocrinology. 106(Suppl.):79.

46. Pieterse, J. R., C. M. Szego, E. B. Pearson, and M. I. Morgan. 1973. Effect of inhibitors of proteolytic enzymes on the growth of normal and polyoma transformed BHK cells. Biochem. Biophys. Res. Commun. 50:354-361.

47. Pieterse, J. R., and C. M. Szego. 1977. Blockade of estrogen uptake and endometrial cell responses to hormones by colchicine and protease inhibitors. Endocrinology. 106(Suppl.):79.

48. Pieterse, J. R., C. M. Szego, and R. E. Seiler. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

49. Pieterse, J. R., C. M. Szego, and R. E. Seiler. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

50. Pieterse, J. R., and C. M. Szego. 1976. Blockade of estrogen uptake and endometrial cell responses to hormones by colchicine and protease inhibitors. Endocrinology. 106(Suppl.):79.

51. Pieterse, J. R., C. M. Szego, and R. E. Seiler. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.

52. Pieterse, J. R., C. M. Szego, and R. E. Seiler. 1976. Lysosomal cathepsin B1: Partial characterization in rat preputial gland and recombinant in response to estradiol-17β. Eur. J. Biochem. 66:453-474.