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SPECIFIC INTERNALIZATION OF ESTROGEN AND BINDING TO NUCLEAR MATRIX IN ISOLATED UTERINE CELLS

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Fractionation of rat uterine cells incubated at 22 °C with 0.2 nM [^{3}H]-estradiol-17β ($E_2\beta$) was performed to analyze the subcellular distribution of internalized hormone. The postnuclear supernatant of homogenates was resolved in Percoll® density gradients into six major fractions defined by enzyme markers. Within 10 s, $E_2\beta$ concentrates at the density of plasma membranes and also at a more buoyant density (ρ = 1.052 ± 0.001) with peak accumulation of hormone by 2 min. Thereafter, binding in the latter fraction declines concomitantly with appearance of a portion of hormone at higher densities corresponding to Golgi and lysosomes. $E_2\beta$ exhibits preferential accumulation in nuclear matrix from 5 to 60 min. Microfiltration and scanning electron microscopy of the buoyant 2-min peak fractions reveal organelles, 50-200 nm. These may represent endocytotic vesicles that serve as vehicles for nuclear transfer of hormone.

Estradiol receptor has widely been considered to be concentrated in the "cytosol" of target cells before exposure to hormone (1-3). However, when account is taken of the conditions of homogenization, saturable binding-sites with high affinity and specificity for estradiol-17β ($E_2\beta$) also occur in plasmalemmal and other particulate fractions of responsive cells (4-9). Interaction of estrogen with such extra- and/or perinuclear macromolecules appears to underlie enhanced binding of the agonist to the cell nucleus (6-10), where the complex is believed to promote expression of the phenotypic effects (1-3). The prominent early association of estrogen: receptor complex with the nuclear matrix of responsive cells may be especially important in promoting the synthesis and processing of polynucleotides (11). Here we show that a substantial proportion of estrogen-binding sites in uterine cells is internalized from plasma membranes in particulate form. These structures appear to serve as vehicles for intercompartmentation of the hormone.

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METHODS

Uteri were excised from rats (85-90/experiment) that had been ovariectomized at 6 wk and maintained for 3 wk in an environment free from steroid hormones (12). Uterine cells were isolated essentially as described before (7). In brief, the organs were finely minced with razor blades at 4 °C and then incubated for 60 min at 37 °C with 2 mg of collagenase (Type I, Worthington, Freehold, NJ) and 1.2 units ml^{-1} of Dispase I (Boehringer-Mannheim, Indianapolis, IN) in Dulbecco's phosphate-buffered saline (DBS). Isolated cells were cultivated 20 h in Minimum Essential Medium with D-valine substituted for L-valine (Grand Island, Grand Island, N.Y.) to limit fibroblast overgrowth (13,14). Media were supplemented with 1×10^{-8}M insulin, 0.5 µg transferrin ml^{-1}, 3.6 µg neutralized selenous acid ml^{-1} (Collaborative Research, Waltham, MA), 1×10^{-9}M cortisol (Schering, Kenilworth, NJ), 100 units penicillin ml^{-1} and 100 µg streptomycin ml^{-1} (Grand Island). The cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. After 20 h, cells were harvested, sedimented by centrifugation at 400 g for 5 min at 4 °C, resuspended in DBS supplemented with 1 mM sodium pyruvate and washed twice by centrifugation. At least 95% of all cells used here excluded 0.05% nigrosin during 5-10 min of incubation in DBS at 22 °C. Homogenization and subcellular fractionation were then carried out as noted below.

RESULTS AND DISCUSSION

Fig. 1 shows an increased accumulation of [^{3}H]$E_2\beta$ by crude nuclei of cells exposed to hormone at 22 °C for 15-60 min as compared with those treated for 10 s to 10 min ($P < 0.001$). Similarly, ultrapurified (15) nuclei and nuclear matrices of uterine cells exposed to $E_2\beta$ at 22 °C, but not at 4 °C, exhibit profound concentration of hormone by 1 h (Fig. 1). It is striking that the nuclear matrix fraction, which constitutes only 4.3% of homogenate protein and 1.4% of total DNA, when equilibrated with $E_2\beta$ 1 h at 22 °C, accounts for ~62% of binding of the ultrapurified nuclei (cf. 11). The essential freedom of these preparations from contamination with marker enzymes from other cellular compartments was verified (12,15; see Fig. 1).

Resolution of the postnuclear supernatant fractions from uterine cells was achieved by centrifugation in Percoll (Pharmacia, Uppsala, Sweden) density gradients. Fig. 2 shows the characteristic distribution of protein and marker enzymes (cf. 16,17).

The intracellular fate of [^{3}H]$E_2\beta$ after its incubation with uterine cells for 10 s-60 min at 22 °C is shown in Fig. 3. Within 10 s, the hormone is concentrated at a density corresponding to plasma membranes

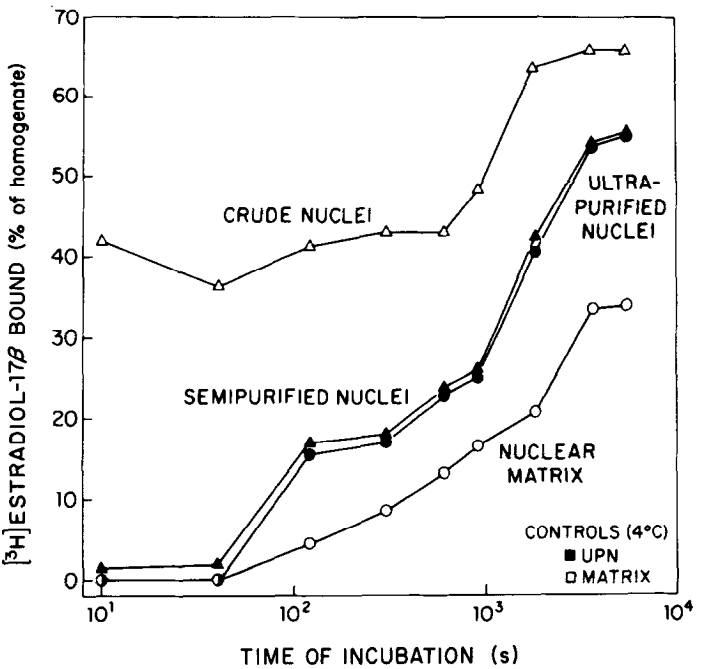

Fig. 1. Distribution of [^{3}H]estradiol-17β in crude and purified nuclei and nuclear matrix subfractions of uterine cells. Cells were treated in vitro with [2,4,6,7,16,17-^{3}H (N)]estradiol-17β ($E_2\beta$; 137 Ci mmol^{-1}, New England Nuclear, Boston, MA) for various times at 22 °C or for 1 h at 4 °C. A low concentration of estrogen, 0.2 nM, was used to minimize nonspecific [^{3}H]$E_2\beta$ binding (cf. 25). After incubation with hormone with stirring for the indicated times, cell suspensions were immediately diluted with 4 volumes of ice-cold DBS and washed 3 times by centrifugation at 400 g for 5 min at 4 °C. Sedimented cells were then suspended in 9 volumes of ice-cold homogenization medium (0.25M sucrose, 0.5 mM $CaCl_2$, 0.5 mM phenylmethylsulphonylfluoride, 5mM Tris-HCl buffer, pH 7.4) supplemented with a 200-fold molar excess of unlabeled $E_2\beta$ (i.e., final concentration 40 nM). The additional estradiol served to reduce inadvertent intracellular binding of hormone during cell disruption (25). Cells were disrupted by the controlled procedures previously validated (7) and fractionated into crude nuclei (△) and postnuclear supernatant essentially as before (7). Semipurfied (▲) and ultrapurified (●; i.e., Triton X-100 treated; UPN) nuclei were prepared as described elsewhere (12,14,15). The latter nuclei were processed for the isolation of nuclear matrix (○; 26). The extent of [^{3}H]$E_2\beta$ binding at 22 °C at times ranging from 10 s to 90 min is given as percent of that in the respective homogenates. Hormone binding at 4 °C in UPN (■) and nuclear matrices (□) is also shown. [^{3}H]$E_2\beta$ at 22 °C in the homogenates was 20,226; 23,692; 24,594 ± 530; 33,881; 30,576; 33,915; 36,961; 38,107; and 37,880 dpm mg^{-1} of protein at 10s; 40s; 2 min; 5 min; 10 min; 15 min; 30 min; 60 min; and 90 min incubation, respectively. At 4 °C, binding of $E_2\beta$ in homogenate was 29,302 dpm mg^{-1} protein after 60 min. Recovery of [^{3}H]$E_2\beta$ binding in crude nuclei and postnuclear supernatant fraction averaged 98.0 ± 1.2%. Analyses of protein were by the method of Lowry et al. (27). Levels of homogenate protein averaged 22.7 ± 1.4 mg/10^8 cells. Recovery of protein in crude nuclei and postnuclear supernatant was 98.8 ± 0.6%. Protein, expressed as % of homogenate, averaged 43.1 ± 1.9, 24.0 ± 1.6, 17.9 ± 0.9 and 3.8 ± 0.2 in crude nuclei, semipurified nuclei, UPN, and nuclear matrix, respectively. Analytical data for enzymes considered extraneous to the nuclear compartment (see text and Fig. 2) for UPN and nuclear matrix, respectively, expressed as % of homogenate were: 5'-nucleotidase (EC 3.1.3.5): 0.5 ± 0.3 (SEM), 0.0 ± 0.0; sialyl transferase (EC 2.4.99.1): 0.2 ± 0.1, 0.0 ± 0.0; acid phosphatase (EC 3.1.3.2): 2.1 ± 0.2, 0.1 ± 0.0; and succinate dehydrogenase (EC 1.3.99.1): 0.1 ± 0.1, 0.0 ± 0.0. (See ref. 14 for comparisons). Mean values are from 1-3 independent experiments.

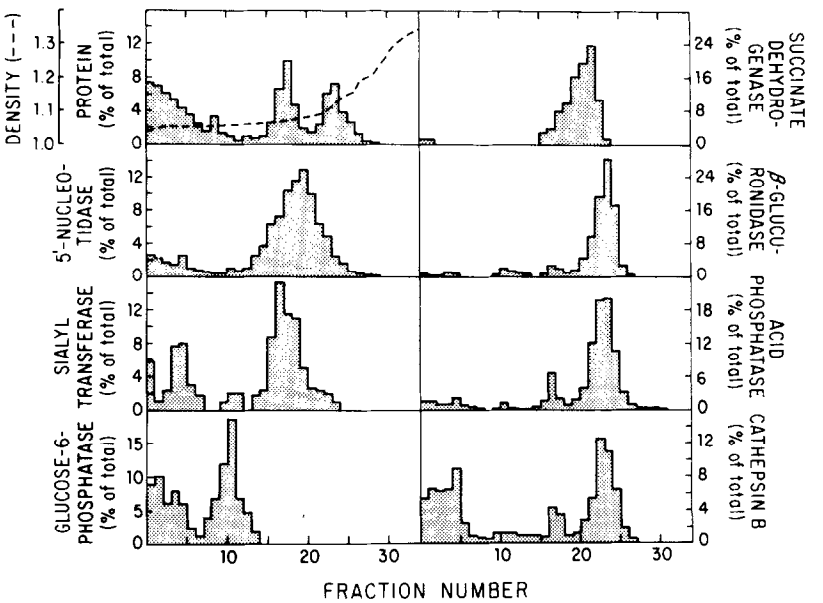

Fig. 2. Subcellular fractionation of postnuclear supernatant in Percoll density gradients. Postnuclear supernatant samples from uterine cells incubated with 0.2 nM [^{3}H]$E_2\beta$ for 2 min at 22 °C (cf. Fig. 1) were centrifuged through a Percoll density gradient (16,28). In 1 x 3.5-inch Sorvall polyallomer tubes, a 4.0 ml cushion of 2.4 M sucrose (ρ = 1.30; ultrapure, Schwarz/Mann, Becton, Dickinson, Orangeburg, N.Y.) was overlaid with 25 ml of Percoll (ρ = 1.065 g ml^{-1}; pH 7.4) and 5 ml of postnuclear supernatant. Centrifugation was for 135 min at 18,000 rpm using a Sorvall SV 288 vertical rotor and RC2-B centrifuge equipped with a rate controller. Fractions of 1.0 ml were collected by upward flow displacement with 2.6 M sucrose. Density, with mean analytical variability of 0.007 units (SEM), was measured as described by Rome et al. (16) and is expressed as g ml^{-1}. Protein (27), with average analytical SEM of 0.7 units, and enzyme activities (14,28), with SEM of 0.5 units were determined as noted. A correction for the potential reactivity or interference of Percoll was obtained in each assay from a sample lacking postnuclear supernatant and centrifuged in parallel (16). Protein and enzyme activities are given as percent of total recovered in each gradient. The enzyme markers utilized were: 5'-nucleotidase (plasma membrane), sialyl transferase (Golgi), glucose-6-phosphatase (EC 3.1.3.9; endoplasmic reticulum), succinate dehydrogenase (mitochondria) and β-glucuronidase (EC 3.2.1.31), acid phosphatase, and cathepsin B (EC 3.4.22.1; lysosomes). Fraction 1 represents the top of gradient.

(1.077 ± 0.003) and also at a more buoyant density (peak 1.050, fraction 5; Fig. 3a). By 40 s, radioactivity in the plasma membrane region is reduced by 72% whereas [^{3}H]$E_2\beta$ binding increases rapidly at the more buoyant density (i.e. 1.052 ± 0.001) to a maximal level at about 120 s (Fig. 3c). Thereafter, hormone binding at 1.049-1.053 g ml^{-1} declines concomitantly with the appearance of a portion of [^{3}H]$E_2\beta$ at densities of Golgi (1.067 ± 0.001) and lysosomes (1.103 - 1.125; Fig. 3 d-g). From 2-60 min, $E_2\beta$ also undergoes transport to the nucleus (cf. Fig. 1). By 60 min,

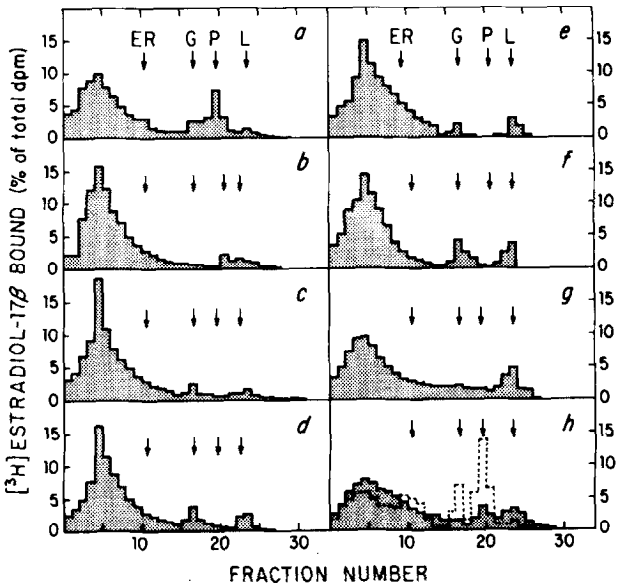

Fig. 3. Distribution of [^{3}H]estradiol-17β binding in postnuclear supernatant fractions of uterine cells in Percoll density gradients. Times of incubation of cells with [^{3}H]$E_2\beta$ at 22 °C were 10 s (a), 40 s (b), 2 min (c), 5 min (d), 10 min (e), 15 min (f), 30 min (g), and 60 min (h). In panel (h), data representing an additional sample exposed to hormone at 4 °C for 60 min are presented as a broken line. In each panel, arrows indicate the predominant locations of marker enzymes (as in Fig. 2) for endoplasmic reticulum (ER), Golgi (G), plasmalemma (P), and lysosomes (L). Levels of bound $E_2\beta$ in each fraction were corrected for free hormone by treatment of samples with dextran-coated charcoal (25). [^{3}H]$E_2\beta$ binding is given as percent of total bound hormone recovered in each gradient. For example, analyses for this variable at 2 min were carried out with an average SEM of 0.5 units. The values for [^{3}H]$E_2\beta$ within the 5-30 min time span, both for the Golgi-marker designated peaks, as well as those for the gradient region maximally enriched in lysosomal enzymes, respectively, were significantly greater than the corresponding data for each organellar peak fraction at the 10s-2min interval (both, $P < 0.001$). A similar degree of statistical significance prevailed when the 10s-2min data for each relevant peak region were compared with those obtained at 5-15 min, or when the latter values were compared with those at the 30-60 min period (all, $P < 0.001$).

[^{3}H]$E_2\beta$ binding in the plasma membrane region of the gradient becomes once more evident (Fig. 3h, solid line). In cells exposed to hormone at 4 °C (Fig. 3h, dashed line), a substantial proportion of estradiol binding remains at the density of plasma membranes.

The $E_2\beta$-binding peak at 2 min (i.e., fraction 5, Fig. 3c) consists of only 2.4 ± 0.5% of homogenate protein but concentrates 10.5 ± 0.6% of total hormone bound (relative specific activity; RSA = 5.15). Fraction 5 also exhibits enrichment of sialyl transferase (RSA = 3.45) and cathepsin B (RSA = 3.29) but not of acid phosphatase (RSA = 1.00), β-glucuronidase (RSA

Table 1. Resolution of [^{3}H]estradiol-17β binding in Percoll peak fraction 5 by microfiltration

Mean pore size (μm)	Protein (% fraction 5)	[^{3}H]E_2β binding (% fraction 5)	(relative specific activity)*
0.050	26.6 ± 2.5 (3)	77.1 ± 10.5 (3)	13.6 ± 1.3 (3)
0.200	4.5 ± 1.1 (3)	22.2 ± 4.3 (3)	28.3 ± 8.5 (3)
0.450	0.2 ± 0.2 (3)	0.4 ± 0.4 (3)	6.1 ± 6.2 (3)

*Relative to whole homogenate (i.e. 24,600 ± 500 dpm . mg protein^{-1})

Filters with a mean pore size of 0.050 μm were from Millipore (Bedford, MA). Those of 0.200 and 0.450 μm were from Gelman (Fisher, Pittsburgh, PA). Filters were exposed to 40 nM unlabeled E_2β for 1 h at 4 °C (to prevent nonspecific adsorption of [^{3}H]E_2β) and washed extensively with Ca^{2+}, Mg^{2+}-free DBS before application of samples. Aliquots of peak fraction 5 (ρ = 1.049-1.053) from cells exposed to E_2β for 2 min at 22 °C (cf. Fig. 3c) were then applied under slightly negative pressure at 4 °C. Filters were washed with 10 volumes of ice-cold Ca^{2+}, Mg^{2+}-free DBS, dried and analyzed. [^{3}H]E_2β binding and protein in peak fraction 5 averaged 641,362 dpm . mg protein^{-1} and 5.70 mg, respectively. Data are given as mean ± SEM(n). Samples of fraction 5 from cells exposed to tritiated hormone for 60 min at 4 °C (cf. Fig. 3h) showed little retention of [^{3}H]E_2β (i.e., 2.7%) on filters of ~0.050 μm.

= 0.16), or 5'-nucleotidase (RSA = 0.73). Further resolution of [^{3}H]E_2β binding in fraction 5 was obtained by microfiltration (Table 1). Significant retention of specifically-bound estradiol was found only on filters with a mean pore size of 0.050 μm and 0.200 μm, yielding enrichment of hormone binding up to 28 times homogenate levels. Similarly, experiments with "cytosol" (cf. 7) after 2 min of cellular exposure to [^{3}H]E_2β at 22 °C, show that 38.8 ± 4.0% of [^{3}H]E_2β and 13.3 ± 1.8% of protein are retained by filters with a mean pore size of 0.050 μm. Preliminary examination of washed filters by scanning electron microscopy (courtesy of C.O. Rambo) revealed numerous ovoid and spherical organelles, 50 - 200 nm.

The present data on the contribution of intracellular particles to the internalization of estradiol by uterine cells (cf. also 5, 18) are consonant with earlier reports on the uptake of aldosterone (19) ouabain (20), and triiodothyronine (21) into target cells after initial binding at the plasmalemma. Extensive additional data summarized elsewhere also suggest that specific entry of estradiol-17β into target cells may occur by receptor-

mediated endocytosis (5,6). The precise nature and distribution of the previously unreported estrogen-binding particles indicated by the present experiments remain to be elucidated. The enzymic profile of the low density fraction differs from that of Golgi, lysosome and plasma membrane fractions. The newly-identified particulate components possess some features in common with endosomes, which concentrate and transport asialotransferrin and insulin (22) and EGF (23). It is notable that the concentration of $E_2\beta$ in the cytoplasmic particles decreases as hormone binding in nuclei and nuclear matrix increases. Taken together with the demonstration that in uterine cells isolated under the strictest estrogen-free conditions, a significant proportion of $E_2\beta$ likewise reaches the Golgi and lysosome compartments before the peak in nuclear accumulation of hormone, these observations may shed further light on the acute phases of estrogen disposition in target cells (6,24).

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