

ESTRADIOL-INDUCED REDISTRIBUTION OF LYSOSOMAL PROTEINS IN RAT PREPUTIAL GLAND

Evidence from Immunologic Probes

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ABSTRACT

The influence of estrogen on the subcellular localization and distribution of lysosomal components of preputial gland was investigated in the ovariectomized rat. Antisera of high titer and specificity toward high-density lysosomal lipoproteins of this organ were raised in rabbits. The immunologic effectiveness of the IgG fraction so obtained was confirmed by microcomplement fixation, immunodiffusion, and immunoelectrophoresis. By both direct and indirect immunofluorescence techniques, cryostat sections of preputial gland from the control animals exhibited pinpoint cytoplasmic fluorescence, of dimensions corresponding to those of lysosomes. In contrast, specific immunoreactive material in corresponding target cells from animals receiving 0.1 μg of estradiol-17 β /100 g body wt only 2 min earlier was distributed more homogeneously, indicating release of antigen from the membrane-bounded organelles. Moreover, specific immunofluorescence became evident at cell surfaces and in peri- and supranuclear localization, sites essentially negative in the controls. These effects were intensified at 15 min, as well as by maximal physiologic dose (0.5 μg /100 g body wt) of hormone. The relatively less active epimer, estradiol-17 α , exhibited only very limited effectiveness by some of these criteria. These observations, taken together with independent biochemical and ultrastructural evidence, lead to the conclusion that structural labilization of lysosomal constituents and their translocation to the nuclear compartment are early correlates of estrogen action.

Available biochemical and morphologic evidence indicates that reduced structural latency of components of lysosomes is a rapid response of specific target cells to certain tropic hormones (see reference 25 for review). This process is accompanied by redistribution of constituents so released to the cell surface (14, 15) as well as their access to the nucleus (27, 29, 31). To extend these observations further, we have analyzed this phenomenon in an estrogen-responsive tissue by immunocytologic methods. By means of specific immunofluorescent probes, the present work demonstrates

that mobilization of lysosomes and redistribution of their antigenic components in preputial gland are early manifestations of the administration of physiologic doses of estradiol-17 β to the ovariectomized rat.

MATERIALS AND METHODS

Animals and Tissue Sources

Sprague-Dawley rats were ovariectomized at approx. 6 wk of age and 150 g body wt. They were then maintained under controlled conditions of light and temperature in a low-steroid environment for 3 wk, as previously

described (24). Such animals, anesthetized lightly with subcutaneous Nembutal (sodium pentobarbital; 5 mg/100 g body wt), either served as a source of tissues for the isolation of antigen, or were used to supply frozen sections for its immunologic localization. In the latter case, the rats were generally injected by saphenous vein either with estradiol-17 β , 0.1 or 0.5 μ g/100 g body wt, or with the corresponding placebo saline control solution (see reference 26). Additional animals received the relatively inactive epimer, estradiol-17 α , by the same route. At 2 or 15 min thereafter, representative 1–2 mm portions of selected tissues were snap-frozen in liquid nitrogen for preparation of cryostat sections as described below. Such tissues included preputial gland, which is exceptionally enriched in lysosomes (see reference 30), uterus, another estrogen-responsive organ, and a number of “control” tissues generally considered inert toward this hormone, i.e. thymus, diaphragm, and lung. In the case of lung, the organ was inflated with Tissue-Tek (Ames Co., Elkhart, Ind.) (19) just before delivery of the segments into liquid nitrogen.

Isolation of Lysosome-Enriched

Preparations

Purified lysosome fractions were isolated from fresh preputial glands that had been pooled from 40 to 90 rats by methods slightly modified from Szego et al. (29, 30). The chilled organs were minced in groups of 15–20 paired glands. Comminution and subsequent preparative procedures were conducted at 0°–4°C. Cellular disruption was carried out by means of a special press (30). In some experiments, rupture of cell membranes was achieved by means of cavitation at 900 lb/in² in a “nitrogen bomb” (Parr Instrument Co., Moline, Ill.). The resultant homogenates were then processed for isolation of lysosome-enriched fractions, essentially free of mitochondrial contamination (30), as indicated above. All reagents, except as noted, were analytical grade.

Separation of Soluble Antigenic Proteins

Purified lysosomes corresponding to LP2 (30) from 20 rats were suspended in 5 ml of a 5-mM sodium phosphate buffer, pH 7.0, also containing 1 mM EDTA and 0.1% (vol/vol) Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.), and exposed to nitrogen cavitation at 1,700 lb/in². The resultant preparations were centrifuged at 105,000 g for 1 h to obtain particle-free lysosomal components generally corresponding to LP2S4 (cf. reference 30). This material was either processed further as indicated below or stored briefly at –80°C until use. Corresponding preparations from lung were also obtained.

Fractionation of Lipoprotein Antigens

Material solubilized from purified preputial gland lysosomes by the procedures described above was treated for separation of lipoproteins by differential flotation

into low-density (LDLP), high-density (HDLP), and very-high-density (VHDLP) fractions by the methods of Goldstone et al. (4). It had previously been shown that the HDLP fraction from rat preputial gland, representing approx. 20% of the nonmembrane proteins of the lysosomal compartment or about 0.6% of total cellular protein, was selectively enriched in components with high-affinity binding properties for estradiol-17 β (Table 2 in reference 25). Proteins from preputial gland lysosomes corresponding to this fraction (PHDLP) were isolated, dialyzed against 5 mM sodium phosphate buffer, pH 7.0, in the presence of 1 mM EDTA, and stored briefly at –80°C until use. A corresponding fraction (LHDLP) from lung, a tissue not normally considered a primary target for estradiol-17 β (see references 22 and 28), was also prepared by parallel methods.

Protein analyses were carried out by the method of Lowry et al. (10). In some cases, the fluorescamine procedure was used (32).

Immunization

Female rabbits of the New Zealand strain and weighing approx. 2.5–3 kg were bled from the central cutaneous artery of the ear for procurement of nonimmune sera (NRS) two days before immunization was begun. All sera were frozen without preservatives at –30°C promptly after blood centrifugation and thereafter thawed only once for further processing or immediate use.

An appropriate amount of antigen, diluted to 1 ml with 0.9% NaCl, was emulsified with an equal volume of Freund's complete adjuvant, using a Virtis homogenizer with a microcup surrounded by plastic bags of ice-salt slurry. Equally distributed volumes of the resultant emulsion were then injected subcutaneously and intradermally at multiple sites, including the footpads and the belly of each of two rabbits. Approx. 120, 100, and 395 μ g of preputial gland HDLP, lung HDLP, and preputial gland LP2S4, respectively, were used for the first course of immunization of each rabbit. Three weeks later, the dosage regime was repeated in Freund's incomplete adjuvant. After 1 wk, the resultant antisera were analyzed for immune titer by complement fixation (see below). Thereafter, antisera were generally collected through weekly bleedings of 30–40 ml, with allowance for occasional rest periods. Except for antisera to lung HDLP (in which case a booster series in Freund's complete adjuvant was required 3 wk after the second course of immunization to yield adequate titer), the antibody levels elicited by the above procedures were substantial (see below) and were so maintained over at least a 20-wk period.

Preparation of IgG

IgG was obtained by precipitation of a crude immunoglobulin fraction with ammonium sulfate, pH 7.0, to 45% saturation (5). The precipitate was dissolved in a minimum volume of PBS₁ (10 mM sodium phosphate

buffer, pH 7.5, containing 15 mM sodium chloride), and the solution was dialyzed against this buffer for 1–2 days or until no further ammonium ions were detectable in the dialysate by Nessler's reagent.

The crude immunoglobulins were then fractionated by application to an *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) column (microgranular size; pre-swollen) which had been equilibrated with PBS₁. During elution with additional buffer, samples of 1 ml were collected, and their OD was read at 280 nm in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) equipped with Gilford attachments (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The peak tubes, consisting of predominantly IgG (3), were pooled and stored in 2 to 5-ml lots at –80°C.

Complement Fixation

Quantitative microcomplement fixation was conducted generally according to the procedures of Levine (9), using a range of concentrations of antigen from 0.01 to 1 µg/ml and serial dilutions of antibody. Sensitized erythrocytes were prepared from sheep cells (Mission Laboratory Supply, Rosemead, Calif.) and rabbit anti-sheep erythrohemolysin (Baltimore Biological Laboratories, Baltimore, Md.) by conventional methods. Guinea pig complement C' was obtained in 1-ml frozen aliquots from Mission Laboratory Supply and stored at –80°C. Complement sufficient for a single analysis was defrosted once only, just before use. Controls included buffer blanks, tubes in the entire range of concentrations in which either antigen or antibody was omitted, and complement blank. Corrections for pro- or anticomplementarity were negligible throughout all antigen concentrations. At levels of antibody greater than 1:1,000, minor corrections for procomplementarity (approx. 5%) were applied.

Immunodiffusion

Double immunodiffusion in a radial pattern was carried out on 1 × 2-inch microscope slides in 1% agarose gels (A-grade; Calbiochem, San Diego, Calif.) prepared in 0.85% sodium chloride, buffered with 10 mM sodium phosphate, pH 7.0, in the presence of 0.02% sodium azide, essentially according to standard procedures (2, 13). The resultant gels were stained with Amido black and photographed on Kodak Panatomic-X film.

Immunoelectrophoresis

Microimmunoelectrophoresis of antigen was conducted on 2 × 3-inch microscope slides in 1% agarose gels in Veronal buffer (sodium diethylbarbiturate; Beckman Instruments, Spinco Div., Palo Alto, Calif.), pH 8.6 and ionic strength 0.075, at a constant current of 8 mA/slide for 2.5 h (2, 13). Thereafter, the troughs were charged with antisera or purified IgG and immunodiffusion was allowed to proceed for 2 days at room temperature in the electrophoresis chamber (Consolidated Labo-

ratories, Inc., Chicago Heights, Ill.). The slides were next washed with 2% NaCl (2 days) and with double-distilled water (1 day), dried, and stained with Coomassie Brilliant Blue. After destaining, the slides were photographed with transillumination on Kodak Panatomic-X film.

Immunofluorescence

Conjugation of purified IgG with fluorescein isothiocyanate (FITC; Calbiochem) was carried out at 0–4°C as previously described (17, 18; cf. also reference 8), using 0.05 mg FITC:1 mg protein. The labeling ratio of the product was generally in the range of 1.23 to 1.30 (OD 495 nm/280 nm). After passage through a G25 Sephadex column to remove unbound dye, the purified conjugated-IgG was absorbed twice with acetone-dried rabbit liver powder (rat liver powder in the case of commercial goat anti-rabbit IgG to be used for indirect immunofluorescence) and clarified by high-speed centrifugation. The resultant supernate was then passed through a 0.45-µm pore filter.

Tissue Sections

Frozen sections of 4 and 5 µm were cut on a Slee cryostat at temperatures ranging from –15° to –25°C. The sections were mounted on cover slips, air-dried briefly at room temperature, fixed for 10 min in acetone (spectrophotometric grade; Matheson Coleman & Bell, Norwood, Ohio), and air-dried.

Staining Procedures

Sections were rehydrated with PBS₂ (10 mM sodium phosphate buffer, pH 7.0, in 150 mM sodium chloride) at room temperature. For the direct method, the sections were incubated with the conjugated antibody or with control solutions for 30 min, followed by four washes of 5 min each with PBS₂. After a brief rinse with distilled water, the slides were air-dried and mounted in Elvanol (Dupont Instruments, Wilmington, Del.). For the indirect method, the procedures were similar, except that the sections were treated first with the unlabeled antibody, washed with two changes of 5 min each with PBS₂, followed by the application of FITC-conjugated goat anti-rabbit IgG (Cappel Laboratories, Downingtown, Pa.). Corresponding sections from a similar series of tissues preserved in Bouin's fixative were stained with hematoxylin and eosin for reference.

Photomicrography

The sections on cover slips were viewed in blind-coded fashion shortly after mounting, with either a Zeiss model RA or a Leitz Ortholux microscope. In the former case, the transmission light source was an Osram HBO-200 mercury-vapor arc lamp; the latter was equipped with a xenon lamp. In either case, a heat-barrier filter, BG38, and the appropriate primary and secondary filters to yield the narrow 490–510 nm range for fluorescein im-

munofluorescence were used. The preparations were photographed with a 40 \times - or 45 \times -objective, generally on Kodak Tri-X film.

RESULTS

Properties of the Antisera

When analyzed by microcomplement fixation, the antibodies raised against preputial gland components were of substantial titer. Thus, Fig. 1 shows that the activities of antisera directed against either total "soluble" proteins of purified lysosomes (PLP2S4) or the high-density lipoproteins (PHDLP) of this organ were demonstrable at dilutions of $>1:1,500$ when challenged by submicrogram concentrations of corresponding antigen. These results were confirmed with analogous preparations from additional rabbits. The semipurified IgG preparations were of comparable effectiveness (data not shown). Negligible amounts of complement were fixed in the absence of antisera. Nonimmune sera were uniformly inactive (Fig. 1, NRS). Moreover, the antisera directed against HDLP of lung and preputial gland, respectively, provoked complement fixation only in the presence of >100 -fold higher concentrations of the inappropriate antigens, thus demonstrating tissue specificity of the antisera obtained.

Further analysis of these preparations for immunologic specificity was carried out by means of radial double immunodiffusion vs. both PHDLP and PLP2S4-antigens, with the results depicted in Fig. 2. Similar patterns of immunoprecipitins re-

sulted in both sets of experiments, with a single line of identity occurring among the crude PHDLP-antiserum, its semipurified IgG, as well as the FITC conjugate prepared therefrom, and the total soluble proteins of purified lysosomes. Once again, NRS was inert. Antiserum directed against the HDLP of purified lysosomes from lung failed to cross-react with either of the antiserum preparations raised against fractions of the purified organelles from preputial gland (Fig. 2). Immunoprecipitin elicited by interaction between PHDLP and the IgG of the corresponding antiserum was abolished by prior treatment of the latter with a crude homogenate of preputial gland, but not by similar preabsorption with a thymus preparation (not illustrated).

These specificities were further characterized by immunoelectrophoresis. As seen in Fig. 3, both the antiserum directed against PHDLP and the IgG prepared therefrom exhibited essentially equivalent reactivity toward the preputial antigen preparation, yielding two major precipitin arcs. These responses were, as anticipated, shared by the total soluble proteins of preputial gland. Thus, although polyacrylamide gel electrophoretic analysis in the presence of sodium dodecyl sulfate had revealed a substantial number of protein components in both LP2S4 and in its lipoprotein subfractions (Fig. 10 A in reference 25), the present immunoelectrophoretic data indicate that two of these predominate as antigens. A high-density lipoprotein fraction of purified lysosomes from lung

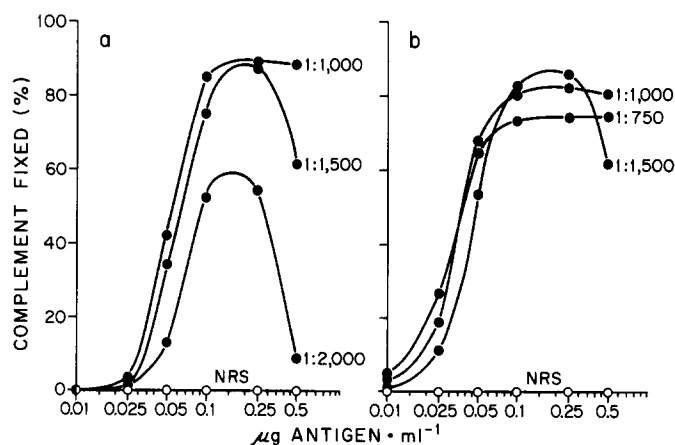


FIGURE 1 Microcomplement titration curves of antisera directed against a high-density lipoprotein fraction (PHDLP; section *a*) and total soluble proteins (PLP2S4; section *b*) of highly purified lysosome preparations from rat preputial gland, as analyzed with various concentrations of corresponding antigen. NRS (open circles), nonimmune rabbit serum. For details, see text.

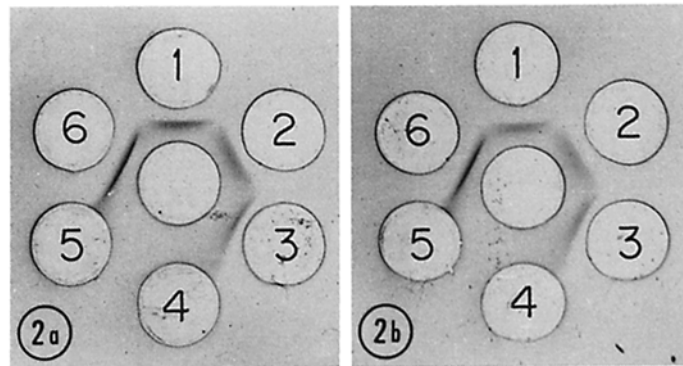


FIGURE 2 Double immunodiffusion of high-density lipoprotein fraction (PHDLP; section *a*, center well) and total soluble proteins (PLP2S4; section *b*, center well) of highly purified lysosomes from rat preputial gland and various preparations of rabbit sera. Volume in all wells was 20 μ l. Concentration of protein in both center wells was 25 μ g/ml. Identical outer wells in sections *a* and *b* were charged as follows: well 1 contained crude antiserum directed against PHDLP, diluted 1:2; well 2, IgG fraction of anti-PHDLP, 4.84 mg protein/ml; well 3, FITC:anti-PHDLP, 1.95 mg protein/ml. The remaining wells contained, respectively, each at 1:2 dilution, a crude antiserum directed against a high-density lipoprotein fraction of purified lysosomes from lung (anti-LHDLP; well 4), nonimmune rabbit serum (NRS; well 5), and antiserum vs. PLP2S4 (well 6). All four precipitin lines show identity; precipitin lines are absent with NRS and anti-LHDLP.

(LHDLP) displayed only a trace of cross-reactivity toward the preputial antiserum (Fig. 3, arrow).

Preputial Gland Histology

Fig. 4*a* presents for reference a typical histological section of preputial gland of the ovariectomized rat. The classic trabecular pattern of arrangement of the parenchymal cells and the occurrence of smaller epithelioid cells interspersed between acini may be noted (cf. reference 11).

Immunocytochemical Localization of Antigen in the Absence of Estrogen

Cryostat sections of preputial gland of ovariectomized rats, whether otherwise untreated (Fig. 4*b*), or injected intravenously (i.v.) with placebo control solution (Fig. 4*c*), were exposed to fluorescein-labeled IgG by the direct method. Such sections exhibited intense pinpoint fluorescence that was dispersed in powdery, punctate fashion through the cytoplasm (Fig. 4*b* and *c*). Staining was especially prominent in ductal cells (Fig. 4*c*). Occasional clusters of granules were also intensely fluorescent. Nuclei were essentially negative (Figs. 4*b* and *c*). The indirect method, using fluorescein-labeled anti-rabbit IgG prepared in the goat, gave similar results (not shown). Since sensitivity of the direct method was satisfactory, the data to be

presented are confined to this method, because of its somewhat better resolving capacity.

Effects of Estrogen

In sharp contrast to these results in the control preparations, i.v. injection of the ovariectomized animals with estradiol-17 β in the amount of 0.5 μ g/100 g body wt at 15 min before excision of the preputial gland invariably resulted in an immunofluorescence pattern that was more generalized (Fig. 4*d* and *e*). A glassy fluorescence was rather homogeneously distributed over the section, with essentially no antigen remaining in particulate form. Fluorescence was especially intense at the luminal surfaces of estrogen-pretreated ductal cells participating in holocrine secretion (Fig. 4*f*). In addition, sections taken from preputial gland of animals exposed to 0.5 μ g of estradiol-17 β for 15 min in vivo were characterized by a ring of especially intense fluorescence surrounding the nuclei, many of which exhibited notable enlargement by the present criteria (Figs. 4*d-f*). In such sections, fluorescence was likewise detectable over and, presumably, within nuclei, phenomena not without precedent in analyses of estrogen-responsive cells by independent morphologic as well as biochemical criteria (see below). These and the further observations to be described, as carried out in the standardized, blind-coded fashion, were con-

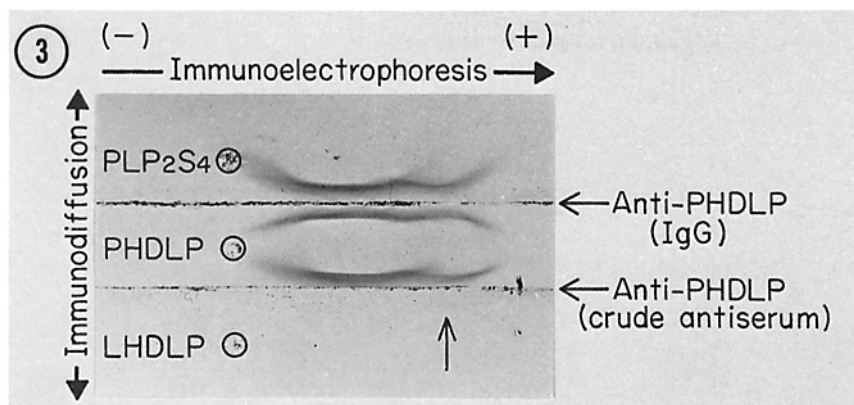


FIGURE 3 Immunoelectrophoretic analysis of preputial-gland and lung antigens. Immunoelectrophoresis on a 2×3 -inch glass slide was carried out in 1% agarose in Veronal buffer, as described in the text. In $2\text{-}\mu\text{l}$ total volume each, the wells contained protein in the following amounts: PLP₂S₄, $3.16\ \mu\text{g}$; PHDLP, $1.82\ \mu\text{g}$; and LHDLP, $1.93\ \mu\text{g}$, respectively. After electrophoresis, $50\ \mu\text{l}$ each of antiserum vs. PHDLP ($3.78\ \text{mg}$ protein) and the IgG prepared therefrom ($0.31\ \text{mg}$ protein) were placed in the indicated troughs. The slide was further processed as described in the text. The dual precipitin arcs reveal the presence of two major antigenic determinants. Only a very faint cross-reaction is seen between the lung antigen and the antiserum directed against the corresponding fraction of the purified lysosomes from preputial gland (arrow).

sistently reproducible in replicate sections of the same tissue, as well as from experiment to experiment.

Dosage and Time-Course

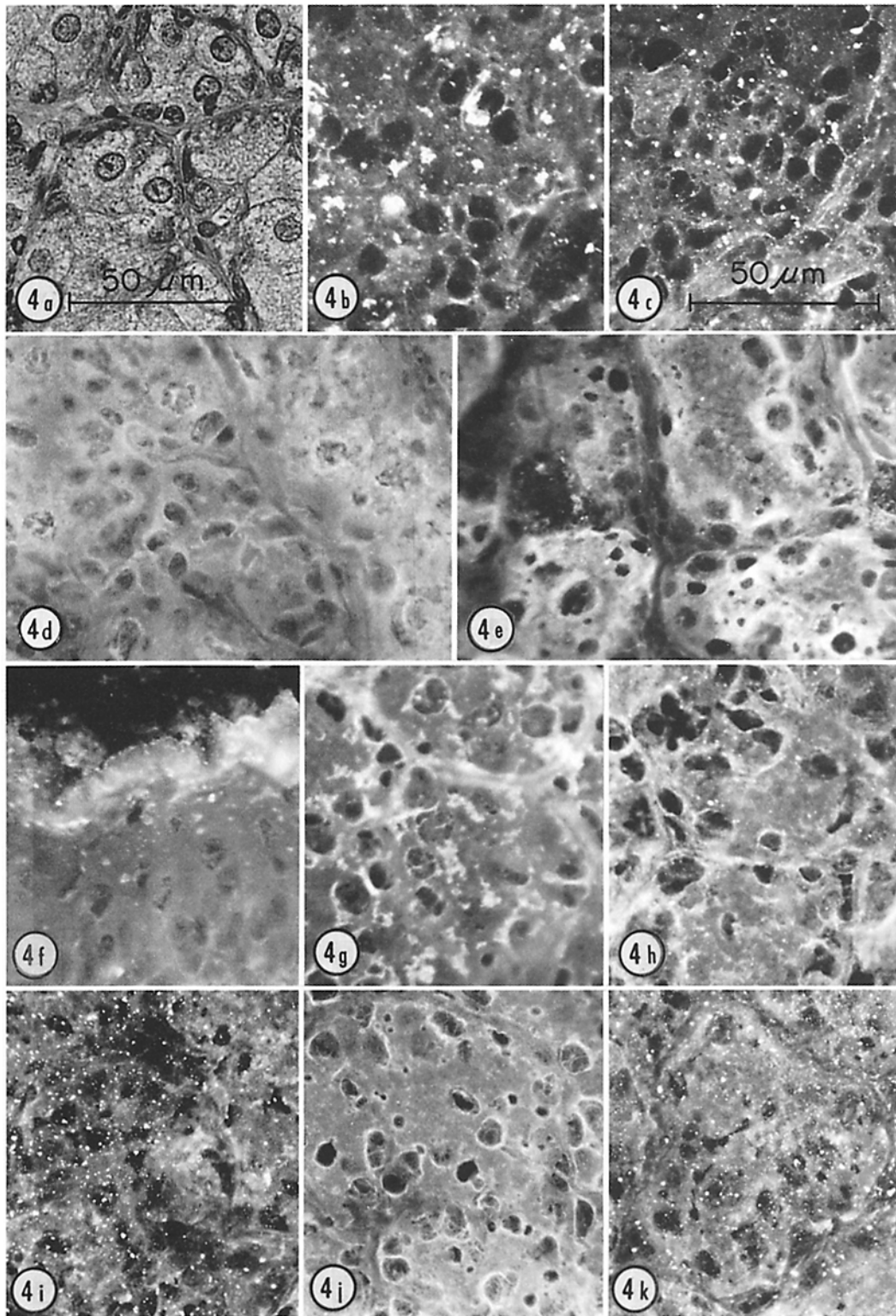
Because this dose of hormone, although considered within the physiologic range, yields maximal responses in sensitive target cells (24), these experiments were repeated at a concentration of only $0.1\ \mu\text{g}/100\ \text{g}$ body wt, a dose essentially twice the minimum effective dose (26). The phenomena described above remained demonstrable, as illustrated by Fig. 4g. Moreover, this latter dose of estrogen was effective, although at a discernibly diminished level, even when allowed to act for only 2 min *in vivo* (Fig. 4h). In this case, submaximal liberation of antigens is denoted by the fraction still in particulate form. Such a rapid time-course of antigen redistribution within the target cell, as well as hormonal sensitivity, are compatible with data obtained from biochemical analyses for lysosomal marker enzymes (23, 25, 29, 31).

In many of these cases, repetition of the experiments with IgG directed against the total soluble proteins of preputial gland (PLP₂S₄) yielded similar data but with somewhat lower sensitivity (not illustrated). Whether this resulted from relatively attenuated content of hormone-binding compo-

nents, as compared with the high-density lipoprotein fraction, has not been determined.

Stereospecificity of Hormone

Estradiol- 17α , the epimer with approx. $1/40$ th the activity of its -17β counterpart by most criteria (see reference 26), acting for 15 min at a dose of either 0.1 or $0.5\ \mu\text{g}/100\ \text{g}$ body wt, exhibited only a barely perceptible capacity to promote redistribution of antigen (Fig. 4i and k). Thus, close inspection reveals a very limited degree of reduction in punctate localization of fluorescence in response to the 17α -steroid. This marginal effect demonstrates the exceptional sensitivity of the present immunocytologic criteria. Biochemical analyses had previously failed to detect augmented liberation of marker enzymes during incubation of lysosomes that had been isolated after *i.v.* administration of the 17α -epimer, as compared with control preparations that, themselves, exhibit autolytic release of hydrolases at a finite rate (29, 30). This was in sharp contrast to the pronounced effect of estradiol- 17β in enhancing such release. However, in the present work, little or no nuclear translocation of antigen was evident at either dose of the relatively inactive congener, especially when compared to the striking effects of the corresponding estradiol- 17β -treated preparation that had been processed within the same



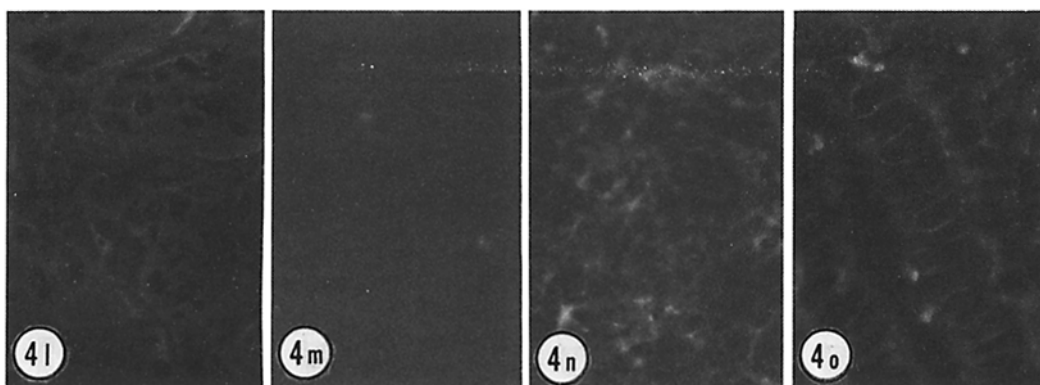


FIGURE 4 Distribution of high-density lysosomal lipoproteins in preputial gland as revealed by direct immunofluorescence. (a) Bouin's-fixed section of preputial gland of ovariectomized rat, stained with hematoxylin-eosin for morphologic reference; $\times 540$. (b) Frozen section of preputial gland of control, uninjected ovariectomized rat, stained with fluorescent antibody to high-density lipoprotein fraction of soluble proteins from preputial gland (PHDLP). Antigen is localized in punctate fashion throughout the cytoplasm; nuclei essentially unreactive; $\times 580$. (c) Frozen section of preputial gland from preparation injected with placebo control solution 15 min before excision of tissue; otherwise, similar to 4b; $\times 580$. (d) Preputial gland of ovariectomized rat injected i.v. 15 min earlier with $0.5 \mu\text{g}$ estradiol- $17\beta/100$ g body wt. Note homogeneous distribution of immunofluorescence resulting from treatment with FITC: anti-PHDLP, both over cytoplasm, at cell borders, as well as over and around nuclei. Some of the latter are appreciably enlarged; $\times 540$. (e) As Fig. 4d; another specimen. Perinuclear fluorescence is especially prominent; $\times 540$. (f) Localization of immunofluorescence over cytoplasm and nuclei of section from another specimen treated as Fig. 4d and e. Intense fluorescent stain, both particulate and diffuse, in epithelial cells bordering glandular lumen, engaged in hormonally accentuated holocrine secretion; $\times 540$. (g) Preparation injected with only $0.1 \mu\text{g}$ of estradiol- $17\beta/100$ g body wt 15 min before tissue excision and treatment otherwise as Fig. 4d-f. Reactions are not so extensive as with maximal dose of hormone; $\times 540$. (h) As Fig. 4g, except that the reduced dose of hormone was allowed to act for only 2 min before rapid removal of tissue for immunocytologic processing. Localization of immunoreactivity closer to that of controls (Fig. 4b and c) but some diffuse staining over cytoplasm and reduction of punctate fluorescence already evident; $\times 580$. (i) Preparation injected with $0.1 \mu\text{g}$ of estradiol- $17\alpha/100$ g body wt 15 min before tissue excision and treatment otherwise as in Fig. 4d-f; $\times 540$. (j) Preparation corresponding to Fig. 4i and k except that pretreatment was with estradiol- 17β . This sample, which was processed simultaneously with i and k to facilitate direct comparison, reveals extensive redistribution of antigen, including nuclear concentration (cf. also Fig. 4d-f); $\times 540$. (k) As Fig. 4i, except that the dose of estradiol- 17α was $0.5 \mu\text{g}/100$ g body wt; $\times 540$. Localization of immunoreactivity after treatment with the 17α -congener (Fig. 4i and k) remains largely punctate, as in controls; however, a very minimal degree of diffuse staining over the cytoplasm is evident, especially in response to the higher dose (Fig. 4k). Moreover, absence of the nuclear translocation response to $0.5 \mu\text{g}$ of estradiol- 17α allowed to act for 15 min in vivo (Fig. 4k) is to be contrasted with the effectiveness of only $0.1 \mu\text{g}$ of estradiol- $17\beta/100$ g body wt, acting for only 2 min (Fig. 4h). (l-o) A series of controls for immunospecificity; all $\times 540$. (l) Control preputial gland treated with FITC:IgG from nonimmune serum. (m) Control preputial gland treated with FITC:IgG from anti-PHDLP that had been preabsorbed with whole homogenate of fresh preputial gland, extinguishing the antigenic staining. (n) Thymus from control ovariectomized animal, processed for direct staining with FITC:IgG from anti-PHDLP as in Fig. 4a-h. Note that only very occasional specific fluorescence, confined to dendritic macrophages, is evident. Neither relative intensity nor localization of immunologic staining, if any, was modified in the corresponding tissues pretreated with estradiol- 17β under the respective experimental conditions shown in Fig. 4l-n (not illustrated). (o) Uterus from ovariectomized rat that had been pretreated with estrogen as in Fig. 4d, then processed as in Fig. 4n. Negligible immunofluorescence, demonstrating lack of overlap of antigenic determinants of the lysosomal complement of an additional target cell for estrogen.

experiment to facilitate direct comparison (Fig. 4j). Such lack of influence of the 17α -compound on nuclear translocation of lysosomal components by the present criteria is in accord with previous findings, obtained by biochemical analyses of ultrapurified nuclei for marker enzymes that are essentially foreign to nuclei under basal conditions (29, 30), or by vital staining of isolated nuclei with acridine orange (28).

Immunologic Controls

Cryostat sections of preputial glands, whether from control, ovariectomized rats or rats treated with estradiol- 17β , invariably failed to yield specific fluorescence when exposed to FITC:IgG from nonimmune sera, as shown by a representative example in Fig. 4l. Moreover, when fluorescein-labeled IgG directed against PHDLP was preabsorbed with a homogenate of preputial gland, its capacity to produce immunocytologic staining was lost (Fig. 4m). In contrast, when such a preparation was preabsorbed with a homogenate of thymus, essentially full activity was retained (not illustrated).

Tissue Selectivity

Fig. 4n reveals that thymus gland from the control ovariectomized rat shows negligible staining with FITC:anti-PHDLP. This minimal reactivity, which was confined to dendritic macrophages, was unmodified by exposure to estradiol- 17β *in vivo* (not shown). Cells of neither diaphragm nor lung exhibited specific immunoreactivity toward FITC:anti-PHDLP; however, their content of autofluorescent elastin fibers limited their usefulness as control preparations. Uterus, which, like preputial gland, possesses lysosomal proteins of high binding-affinity toward estradiol- 17β (6), showed no cross-reactivity with anti-PHDLP by the present criteria, with or without hormonal pretreatment (Fig. 4o).

DISCUSSION

Recent investigations suggest that limited compartmentation of lysosomal constituents to sites including the plasmalemma and the nucleus may serve as an early step in the coupling of metabolic responses leading to hormone-induced growth and/or differentiation of specific target cells (24, 25, 27, 29, 31). These observations were based in part upon enzymatic analyses of highly purified subcellular fractions. The present experiments

were undertaken in order to test the above-mentioned hypothesis by independent methods that circumvent the need for extensive cellular manipulation. As a result of application of immunofluorescence localization techniques to cryostat sections, it has now been determined that mobilization and redistribution of lysosomal components are indeed early correlates of the presence of physiological concentrations of specific tropic hormone in the estrogen-responsive cells of the rat preputial gland.

Immunization of rabbits with total nonmembrane proteins of highly purified lysosomes from rat preputial gland, or with a high-density lysosomal lipoprotein fraction previously shown to be enriched in components possessing high-affinity binding sites for estradiol- 17β ([Table 2 in reference 25]¹ and footnote 2) led to the production of antisera of substantial titer, as judged by micro-complement fixation. There was negligible cross-reactivity of these antisera with corresponding antigenic preparations from lung, an organ generally considered lacking in estrogen responsiveness, when these preparations were analyzed by complement fixation, Ouchterlony double-diffusion, or immunoelectrophoretic techniques.

Application of FITC-conjugates of the IgG preparations directed against the preputial gland to cryostat sections of this organ taken from control ovariectomized rats demonstrated the requisite sensitivity and specificity. Rigorous controls, including lack of staining with FITC-IgG from nonimmune serum and extinction of immunofluorescence by preabsorption of the effective conjugated IgG with excess antigen, established the immunologic specificity of the reaction observed.

Specific immunofluorescence in the preputial gland of the ovariectomized rat given placebo control solution intravenously was sharply localized to the cytoplasm, with none visible in or over the

¹ Horton, M. J., and C. M. Szego. Manuscript in preparation.

² Experiments have been conducted on cells isolated from preputial gland of ovariectomized rats to determine specific interaction with estradiol- 17β by conventional techniques (34). Specific binding of hormone over a range of physiological concentrations was reduced to $65 \pm 5\%$ (SEM.; $n = 6$) of controls ($P < 0.001$) by addition of $10 \mu\text{l}$ of antiserum directed against PHDLP to the 5-ml incubation medium. In contrast, nonimmune serum or antiserum against the corresponding fraction of lung were ineffective. Pietras, R. J., and C. M. Szego. Manuscript in preparation.

nuclear compartment. Such immunofluorescence was predominantly punctate, giving a powdery appearance to the cytoplasm, of dimensions compatible with those of lysosomes of the same organ viewed after intravital staining with acridine orange (28), and thus just at the limit of resolution of the light microscope.

The character and distribution of specific immunofluorescence in the preputial gland of the ovariectomized rat was radically modified within 2 min of the i.v. administration of physiologic doses of estradiol-17 β . Localization of antigen was much less pinpoint and more diffuse, giving rise to a rather glassy, homogeneous appearance of the section. This response was graded, with respect to both dose of hormone and duration of its action. Thus, exposure of the tissue in vivo to estradiol-17 β at a maximal physiologic dose of 0.5 μ g/100 g body wt for 15 min led to the essential disappearance of all punctate fluorescence in the cytoplasm and dispersion of apparently equivalent immunoreactivity throughout the section, including transfer to the cell surface, which is receiving increasing attention as a site of recognition for estrogen (16) and other mitogenic signals (see references 1 and 20). These responses, which are interpreted to mean escape of lysosomal antigen from the organelles, were especially prominent in ductal regions, known to be particularly sensitive to hormonally accentuated remodeling and rich in secretory product.

Perhaps the most striking feature of antigen redistribution under hormonal influence was the regularity with which intensely stained, immunoreactive material, both particulate and diffuse, appeared in perinuclear rings and also was distributed over, and apparently within, the nuclear compartment. This phenomenon, which confirms and extends prior observations on a limited degree of nuclear invasion of lysosomal components by biochemical (23, 27, 29, 31) and ultrastructural (21) procedures in hormone-activated cells, was a consistent feature of the estrogenic response. In contrast, nuclear translocation of antigen was undetectable by the present criteria after administration of the relatively inert epimer, estradiol-17 α . The latter compound provoked only a marginal degree of release of antigen from particulate form. Such evidence of hormonal specificity gives further support to the physiologic relevance of the present observations.

The immunocytologic correlates of treatment with active estrogen were tissue-selective. Thus,

frozen sections of neither thymus, lung, nor diaphragm exhibited specific immunofluorescence with FITC-conjugated IgG directed against preputial gland HDLP. The data, revealing only a very limited affinity of soluble HDLP from lysosomes of lung toward antibody raised against a similar class of proteins from lysosomes of preputial gland, indicate that lysosomal proteins of the two organs are dissimilar in antigenic determinants. More significantly, the lack of immunofluorescence of uterus when frozen sections were challenged by the direct application of fluorescent IgG that had been elicited by the preputial gland antigen demonstrates that there is no overlap in immunoreactivity, even between two such estrogen-responsive organs. The relative immunologic specificities of these dissimilar structures demonstrate that lysosomal composition of the several organs is grossly divergent.

The present results are in harmony with the evidence available from biochemical methods that lysosomal activation, including penetration of a limited proportion of their marker enzymes into the nuclear compartment, is an early correlate of the application of estrogen in responsive cells. Recently, Wildenthal et al. (33) have demonstrated, by immunofluorescent staining for rabbit cathepsin D activity, that this proteinase, which was essentially confined to lysosome-like particles in the cardiac myocytes of control animals, exhibited more diffuse cytoplasmic staining as well as intense concentration, both particulate and diffuse, in nuclear regions of comparable animals after 3 days of starvation. These observations are strongly suggestive of a parallel to the present data, despite differences in the provocative stimuli that led to lysosomal activation.

Controlled access to the nuclear compartment by lysosomal hydrolases as well as acidic glycolipoprotein matrix may have profound implications for the triggering of genic derepression, as considered in detail elsewhere (24, 25, 29). Preliminary data, obtained by microcomplement fixation and enzymatic analyses of subfractions of ultrapurified nuclei (27, 31) from preputial gland, reveal that lysosomal antigen as well as cathepsin B1 activity are present in the chromatin and in its nonhistone proteins within minutes of exposure in vivo to physiological concentrations of estradiol-17 β .³ These experiments extend evidence already avail-

³ Horton, M. J., C. M. Szego, M. B. Nazareno, and B. J. Seeler. Manuscript in preparation.

able on rapid modification by this hormone of the qualitative and quantitative pattern of nonhistone nucleoproteins of preputial gland, some of which bear striking resemblance to acidic proteins of the lysosomal sap (7). Collectively, these data suggest that re-compartmentation of specific lysosomal constituents in target cells may contribute to the genic derepression (12) that is integral to full expression of the estrogenic response.

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