CONCEPTS RELATED TO SALT RESISTANT ESTRADIOL RECEPTORS IN RAT UTERINE NUCLEI: NUCLEAR MATRIX

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SUMMARY

When ^{3}H-estradiol (0.1 μg) is injected into immature female rats, virtually all of the label that is recovered with uterine nuclei can be solubilized by 0.6 M KCl. Salt resistant uterine nuclear estrogen binding sites do not become labeled within one hour after the injection of ^{3}H-estradiol, but these sites do exist and can be revealed when isolated nuclei are subjected to an *in vitro* estradiol exchange assay. These saturable, high affinity salt resistant sites appear to be associated with the uterine nuclear matrix, a residual structure of the nucleus.

INTRODUCTION

Clark and Peck (1), Baudendistel and Ruh (2,3) and Mester and Baulieu (4) have reported the presence of two types of estrogen receptors in immature rat uterine nuclei. In addition to the well known 0.6 M KCl extractable receptor, they find another estrogen receptor complex which is not extracted by high salt. It has been suggested that long term nuclear retention of these salt resistant complexes is essential for true uterine growth (1,2,3). In contrast, Juliano and Stancel (5) have questioned the concept of two distinct classes of nuclear binding sites. Wotiz and his colleagues (6) also have questioned the separate identity of nuclear salt resistant estrogen binding sites in the uterus and believe that these represent salt extractable receptor trapped within the gelatinous DNA pellet.

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Our analysis of this problem has revealed that methodological differences may account for these divergent interpretations. Under appropriate conditions, salt resistant nuclear estrogen binding sites can be revealed and it appears that these sites may be associated with the nuclear matrix, a residual structural framework of mammalian nuclei.

MATERIALS AND METHODS

Isolation of Crude Nuclei. Immature female Sprague Dawley rats (21-25 days old) received a single subcutaneous injection of either 0.1 μg ^{3}H-17β-estradiol (^{3}H-E_2; [2,4,6,7-^{3}H(N)]-Estradiol, 102 Ci/mmole, New England Nuclear, Mass.), or 0.1 μg unlabeled 17β-estradiol (Steraloids, N.H.), in 0.5 ml of 0.9% NaCl, or vehicle alone. One hour later, the uteri were removed, rinsed in cold TE buffer (10 mM tris HCl, 1.5 mM EDTA, pH 7.4 at 22°C) and homogenized at 4°C in TE buffer (2 ml/uterus) in an all glass Kontes Duall homogenizer with a motor driven pestle. The crude nuclear myofibrillar pellet (780 x g, 10 min) was washed 3 times and resuspended in TE buffer (2 ml/uterus). Nuclei were processed according to the scheme outlined in figure 1.

KCl Extraction. Where indicated nuclei were extracted at 0°C for 30 min in 0.6 M KCl containing 10 mM tris, pH 8.0 at 22°C, to yield a salt soluble fraction and a salt insoluble pellet (780 x g, 10 min). E_2 binding in these fractions was measured either by direct scintillation counting or by the ^{3}H-Estradiol exchange assay.

^{3}H-Estradiol Exchange Assay. When no radioactive label was injected *in vivo*, high affinity E_2 binding sites were quantitated by the ^{3}H-E_2 exchange assay *in vitro* (7). Samples (0.25 uterine equivalents, in triplicate) were incubated in TE buffer (0.7 ml total) at 37°C for 30 min with 14 nM ^{3}H-E_2 (for total binding) or 14 nM ^{3}H-E_2 plus 1.4 μM unlabeled E_2 (for nonspecific binding). Particulate samples (total nuclei or KCl insoluble fraction) were pelleted at 0°C, washed 3 times with TE buffer (780 x g, 10 min, 0°C), and then either dissolved and counted, or KCl extracted before counting. ^{3}H-E_2 exchange on KCl soluble extracts was terminated by the removal of unbound and loosely-bound hormone with dextran-coated charcoal (8); an aliquot of the supernatant was counted.

Liquid Scintillation Counting. Pellets or suspensions were dissolved in 1 ml of Soluene-350 (Packard) and counted in 15 ml of LSC-Complete (Yorktown Res.). KCl extracts (0.5 ml of 1 ml total) were counted in 10 ml of Hydromix (Yorktown Res.).

Isolation of Uterine Nuclear Matrix. Nuclei were processed for the isolation of nuclear matrix essentially as described by Berezney and Coffey (9,10). Crude nuclei were sequentially extracted with 1% Triton X-100 (0°C, 10 min, in 0.25 M sucrose containing TM buffer [10 mM tris, pH 7.4, 5 mM $MgCl_2$]; pancreatic DNase I and RNase A (Worthington; each at 10 μg/ml in TM buffer, 0°C, 30 min); 0.2 mM $MgCl_2$, 10 mM tris, pH 7.4 (LM buffer); 2 M NaCl in LM buffer (2 extractions, 0°C, 30 min); and finally washed and resuspended in TE buffer. (All centrifugations were at 780 x g, 15 to 30 min).

Abbreviation : E_2 = 17β-estradiol = estra-1,3,5(10)-trien-3,17β-diol

RESULTS AND DISCUSSION

One hour following the injection of radiolabeled estradiol into an immature female rat, 210 ± 15 femtomoles of estrogen are recovered with the isolated uterine nuclei (Table 1, I-A). Less than 10% of the total label remains in the nucleus following 0.6 M KCl extraction (I-C). This is in agreement with the observation of Muller et al. (6) who have concluded that salt resistant receptors are an insignificant nuclear component. However, when we further subject the salt resistant pellet to an in vitro exchange assay in the presence of ^{3}H-estradiol an additional 200 fmoles of binding sites are detected (I-D). Approximately 80% of this bound estrogen remains resistant to a second KCl extraction. In summary, labeled estradiol injected in vivo does not appear with the salt resistant sites, but these sites can be revealed when the isolated nuclei are subjected to exchange in vitro.

Additional insight into the apparent difference between labeling binding sites in vivo or in vitro was obtained by injecting an identical amount (0.1 μg) of unlabeled estradiol into immature female rats and then subjecting the isolated nuclei to an exchange assay with ^{3}H-estradiol. The total amount of labeled estradiol bound to the isolated nuclei as revealed by such an in vitro exchange assay was 483 ± 19 fmoles per uterus (II-F). When these in vitro labeled nuclei are subsequently extracted with 0.6 M KCl, 220 ± 10 fmoles of ^{3}H-estradiol (45% of the total nuclear binding) remain in the salt insoluble fraction (II-H). Comparable results are obtained if the experiment is carried out in the reverse order; that is, if the unlabeled nuclei first are extracted with 0.6 M KCl and the salt resistant pellet is then subjected to an exchange assay with ^{3}H-estradiol (II-D). This latter approach is similar to that used by Clark and Peck (1).

Total nuclear estrogen binding sites measured by the exchange assay on isolated nuclei were 2.3 fold more numerous than those found following injection of label in vivo (II-F vs I-A). This increase can be accounted for by the ability of the exchange procedure to detect salt resistant sites in

TABLE 1. Subnuclear Distribution of Specific Estradiol Binding Sites in Immature Rat Uteri Under Various Conditions.[a]

GROUP	SAMPLE	SPECIFIC ESTRADIOL BINDING (fmoles/uterus)[c]	95% CONFIDENCE LIMITS	NO. OF EXPTS.[b]
I. ^{3}H-Estradiol in vivo	Not Exchanged			
A.	Total Nuclei	210 ± 15	179-240	10
B.	Salt Soluble	214 ± 18	178-240	10
C.	Salt Insoluble	22 ± 4	15-29	8
D.	Salt Insoluble, Exchanged	199 ± 20	159-238	5
II. Unlabeled Estradiol in vivo				
	Exchanged, then Extracted			
F.	Total Nuclei	483 ± 19	446-520	10
G.	Salt Soluble	269 ± 33	204-335	5
H.	Salt Insoluble	220 ± 10	201-238	5
	Extracted, then Exchanged			
E.	Salt Soluble	181 ± 20	142-221	3
D.	Salt Insoluble	210 ± 17	176-243	8
III. Saline in vivo				
	Exchanged, then Extracted			
F.	Total Nuclei	299 ± 10	280-318	5
G.	Salt Soluble	133 ± 7	120-146	5
H.	Salt Insoluble	156 ± 11	135-177	7

[a] Refer to figure 1.

[b] Number of separate experiments. Each experiment was performed on uteri pooled from 3-8 animals. Within each experiment all measurements were performed in triplicate.

[c] fmoles/uterus, mean of 3 to 10 experiments ± standard error of the mean.

Significant difference between the means				No significant difference			
I-A	vs	II-F,	$p < 0.001$	I-B	vs	II-G,	$p > 0.1$
I-C	vs	II-H,	$p < 0.001$	I-D	vs	II-D,	$p > 0.2$
III-F	vs	II-F,	$p < 0.001$	II-G	vs	II-E,	$p > 0.1$
III-G	vs	II-G,	$p < 0.01$	II-H	vs	II-D,	$p > 0.2$
III-H	vs	II-H,	$p < 0.01$				

Statistical analysis performed by Student's t test designed to accept unequal size groups.

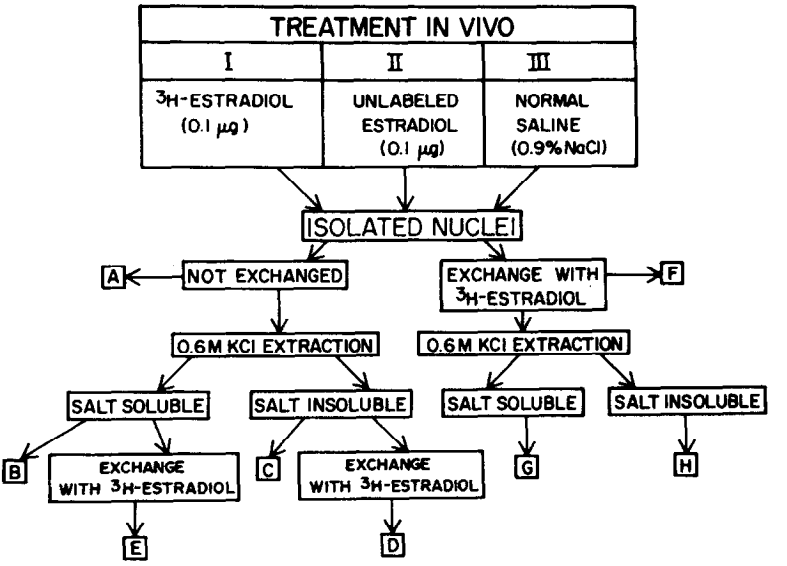

FIGURE 1. Schematic for Table 1.

vitro which are not labeled by the in vivo approach (220 ± 10 fmoles, II-H; vs 22 ± 4 fmoles, I-C; $p<0.001$). In contrast, labeling of the salt soluble fraction is equally efficient by both in vivo and in vitro methods (214 ± 18 fmoles, I-B; vs 269 ± 33 fmoles, II-G; $p>0.2$).

The presence of nuclear estrogen binding sites in uteri from immature animals which have not received exogenous estrogens has been demonstrated by the ^{3}H-estradiol exchange assay (Table 1, Group III; also see refs. 1,2,3,5,7). This observation has often been interpreted to indicate the presence of low levels of endogenous estrogens in immature females (1,2,3,5,7), however, a cause and effect relationship has not been established.

This study has demonstrated the presence of salt resistant uterine nuclear estrogen binding sites under a variety of conditions. It has been postulated that this nuclear component may represent entrapment of the salt soluble fraction in the gelatinous DNA pellet that forms when uterine nuclei are incubated in 0.6 M KCl, and that repeated extraction might remove these "insoluble" sites (6). We have determined, however, that the observation of

salt resistant sites does not result from incomplete extraction. When uterine nuclei, labeled by ^{3}H-estradiol exchange *in vitro*, are treated with DNase I (10 μg/ml, 0°C, 30 min), subsequent KCl extraction (0.6 M) allows the solubilization of 91% of the total nuclear DNA, and no gel is formed; nevertheless, 50% of the total nuclear estradiol binding sites remain in the salt insoluble pellet. A similar number of nuclear binding sites is resistant to extraction by 0.6 M KCl when uterine nuclei are not pretreated with DNase (Table 1, II-H).

The presence of salt resistant estradiol binding sites in uterine nuclei which are essentially devoid of DNA following extraction with a high concentration of salt (0.6 M KCl) suggested the possibility that residual nuclear elements might be involved in salt resistant binding. We have reported that detergent treated nuclei which are extracted with 2.0 M NaCl and treated with DNase and RNase yield a residual nuclear structure which forms a framework for the nucleus; this structure, termed the nuclear matrix, is devoid of over 99% of total nuclear DNA, RNA, and phospholipid, and represents only 5 - 10% of the total nuclear proteins (9). The matrix consists primarily of three acidic polypeptide fractions with molecular weights of 60-70,000, and electron micrographs reveal that the structure is composed of residual elements of the nuclear membrane, nucleolus and interchromatinic structures (10). Furthermore, it appears that the nuclear matrix may be associated with newly synthesized DNA in the regenerating rat liver (11). The isolation, structure and function of this matrix has been reviewed recently (12). We have subjected isolated, crude uterine nuclei to a series of extraction procedures which had been utilized previously to yield the liver nuclear matrix, and our preliminary studies indicate a similar structure in the rat uterus. This uterine matrix preparation was obtained after the following series of nuclear extractions: 1% Triton X-100; DNase treatment; low magnesium; two extractions with 2.0 M NaCl; and two washes with Tris-EDTA buffer. The final uterine nuclear matrix remained after removal of greater than 99% of the total nuclear DNA. This residual nuclear structure was subjected to saturation analysis under exchange conditions

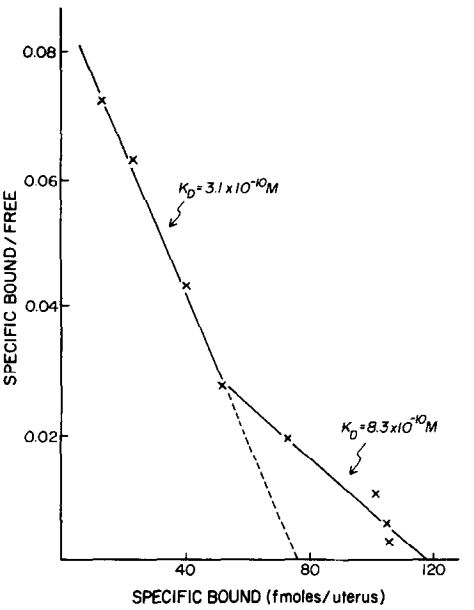

FIGURE 2. Scatchard plot of estradiol binding to isolated uterine nuclear matrix fraction prepared from crude uterine nuclei. Uteri were excised and processed 1 hour after injection of 0.1 μg unlabeled estradiol into 23 day old rats. Nuclear matrix (0.25 uterine equivalent/tube) was incubated at 37°C, 30 min in the presence of ^{3}H-estradiol (concentration range, 7×10^{-12} M to 14×10^{-9} M) without (to measure total binding) or with 1.4×10^{-7} M unlabeled estradiol (to measure nonspecific binding).

and the resulting Scatchard plot is presented in figure 2. High affinity estradiol binding was detected, with apparent equilibrium dissociation constants in the 10^{-10} M range. In contrast, when purified nuclei or nuclear matrix prepared from livers of the same rats were carried through a similar analysis, no high affinity estradiol binding was detected.

The present data would seem to indicate that nuclear salt resistant binding sites in the uterus cannot be occupied by ^{3}H-estrogens within one hour after the injection of label into an immature female. Similar results are obtained at six hours (data not shown). It is not yet clear when or if these sites are occupied in vivo by endogenous estrogen.

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