J. Steroid Biochem. Molec. Biol. Vol. 39, No. 1, pp. 131–132, 1991
Printed in Great Britain

0960-0760/91 $3.00 + 0.00
Pergamon Press plc

SHORT COMMUNICATION

LOCALIZATION OF THE ESTROGEN RECEPTOR IN UTERINE CELLS BY AFFINITY LABELING WITH [^{3}H]TAMOXIFEN AZIRIDINE

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(Received 7 January 1991)

Summary—The possibility that estrogen receptors may exist in uterine plasma membranes was investigated by covalent labeling of estrogen receptors in mouse uterine cells with [^{3}H]tamoxifen aziridine (TA). Isolated epithelial and stromal cells of immature mice were incubated with [^{3}H]TA in the presence or absence of unlabeled tamoxifen, homogenized and separated into nuclear, cytosolic and microsomal fractions by differential centrifugation. These fractions were subjected to SDS-polyacrylamide gel electrophoresis and the proteins labeled covalently with TA were visualized by autoradiography. Proteins labeled specifically with [^{3}H]TA were observed almost exclusively in the nuclear fraction of both epithelial and stromal cells. In contrast, very little labeled protein was detected in the cytosolic or microsomal fraction. Although these data do not preclude the possibility that estrogen binding sites are present in plasma membranes of uterine cells, this cellular fraction is definitely not labeled to a significant extent by [^{3}H]TA. Thus, if membrane estrogen binding sites exist, their structural conformations may be different from that of nuclear estrogen receptors.

Although localization of estrogen receptors (ER) in cell nuclei has been well characterized, the existence of alternative binding sites is more controversial. Evidence in support of estrogen binding sites in uterine plasma membranes, as assessed by classical binding studies, has been reported [1]. However, a similar study determined that membrane binding sites for estradiol were actually cytosolic proteins which contaminated the membrane fraction [2]. Membrane binding sites for estrogens have also been reported to exist in breast cancer cells [3] and brain tissue [4]. The presence of such binding sites could account for a growing number of observations of non-genomic effects of estrogens as reviewed by Weiss and Gurpide [5]. These effects include alterations of membrane permeability and calcium flux in uterine cells, direct effects on plasma membrane bound enzymes (e.g. adenylate cyclase), and alterations in pituitary dopamine receptor binding. Within minutes of administration, estrogens potentiate excitatory amino acid receptor-mediated responses in the cerebellum which has a low density of ER [6]. Thus, plasma membrane binding sites could play a role in the actions of estrogens in addition to those mediated by nuclear ER. The purpose of this study was to determine the cellular localization of specific estrogen binding sites in uterine cells using [^{3}H]tamoxifen aziridine (TA) which covalently labels these receptors [7].

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Epithelial and stromal cells were isolated from uteri of 17-day-old female mice as described previously [8]. Briefly, uteri were removed, bisected longitudinally, and the epithelial or stromal cells were dissociated from the tissue by successive incubations at 37°C in Ca- and Mg-free PBS pH 7.4, which contained 0.1% trypsin or 0.05% trypsin/53 μM EDTA, respectively. Soybean trypsin inhibitor (0.1%) was added after each incubation. Specific estrogen binding sites were labeled by a previously described method [9]. The cells were incubated with or without 6 μM unlabeled tamoxifen (Steraloids, Wilton, NH) in phenol red-free DMEM/F-12 media containing 3% dimethylformamide for 60 min at 4°C followed by addition of [^{3}H]TA (30 nM final; NEN-Dupont) and further incubation for 60 min at 25°C. Competition with unlabeled diethylstilbestrol yielded similar results to those obtained with unlabeled tamoxifen. Cells were washed with DMEM/F-12 and homogenized in buffer containing 5 mM Tris–HCl, 0.25 M sucrose, 0.5 mM $CaCl_2$, 20 μg/ml PMSF, 10 μg/ml leupeptin, and 100 KIU/ml aprotinin. A similar buffer was used by Pietras and Szego [1] to isolate plasma membranes containing estrogen binding sites. Cells were homogenized with a battery-driven pellet pestle (Kontes) for 40 s which disrupted at least 95% of the cells as monitored by trypan blue exclusion. The homogenates were centrifuged at 800 *g* for 10 min at 4°C to collect the nuclear fraction and the supernatant was centrifuged at 110,000 *g* for 45 min at 4°C to separate cytosolic and microsomal fractions. Cytosolic proteins were precipitated with cold acetone for 12 h at −70°C.

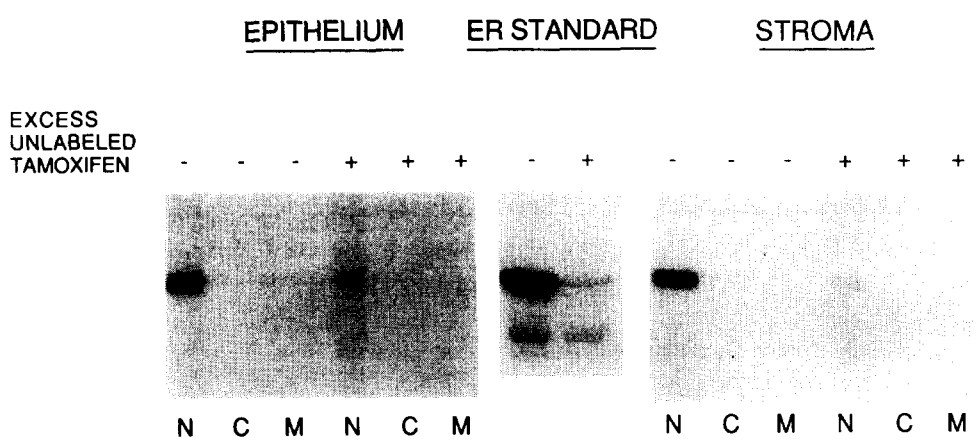

Fig. 1. Proteins of the nuclear (N), cytosolic (C) and microsomal (M) fractions from 1.4×10^7 epithelial or stromal cells which had been labeled with 30 nm [^{3}H]TA in the presence or absence of 6 μM unlabeled tamoxifen were separated on SDS–polyacrylamide gels which consisted of an 8% resolution gel and a 3% stacking gel. The lanes marked standard represent the positive control for ER described in the text. Both labeled and unlabeled tamoxifen were dissolved in ethanol and the final ethanol concentration in the incubations was 5%. Before application to the gel, samples were denatured with 180 mM dithiothreitol and heated to 100°C for 2 min followed by treatment with 0.13 M iodoacetamide and 0.075 M Tris base. The gels were stained with Coomassie brilliant blue, fixed, and soaked in Enlightening (NEN–Dupont) prior to being dried and exposed to Kodak XAR-5 X-ray film.

Proteins in each of the fractions were solubilized in 10% SDS and separated on 8% polyacrylamide gels. ER extracts of whole uteri that had been labeled with 30 nM [^{3}H]TA in the presence and absence of 6 μM unlabeled tamoxifen served as positive controls [10]. Autoradiography of the uterine standard resulted in a labeled protein migrating with a M_W of 65 kDa which was confirmed to be ER by Western blot using H222, an ER monoclonal antibody (courtesy of Dr Chris Nolan, Abbott Labs—data not shown).

The results show that protein specifically labeled with [^{3}H]TA which migrated with the positive control for ER was localized only in the nuclear fraction of both epithelial and stromal cells (Fig. 1). The labeled nuclear protein occurred as doublet which is consistent with previous studies of uterine nuclear ER forms [10]. No label was detected in either the cytosolic or microsomal fractions. In an identical experiment, however, labeled protein which represented a small percentage of that observed in the nuclear fraction was detectable in the cytosol of both cell types and in the epithelial microsomal fraction (data not shown). Such a small amount of labeled protein could reflect an artifact produced by homogenization. We conclude from these results that the microsomal fractions of isolated uterine cells are not covalently labeled by [^{3}H]TA to a significant extent. Thus, estrogen receptors with biochemical characteristics of classical nuclear receptors probably do not exist in uterine plasma membranes. However, these data do not preclude the existence of membrane receptors with which estrogens could interact. Clearly, several investigators have described plasma membrane binding sites [1, 3, 4] and non-genomic actions [5] for estrogens. The data presented herein might indicate that plasma membrane estrogen binding sites differ in structure from nuclear receptors or that estrogens interact with other classes of membrane receptors which TA does not covalently label.

Acknowledgement—Diane M. Ignar-Trowbridge was supported by a PRAT fellowship from NIGMS.

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