J. steroid Biochem. Vol. 36, No. 6, pp. 703–705, 1990
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0022-4731/90 $3.00 + 0.00
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SHORT COMMUNICATION

PREFERENTIAL NUCLEAR BINDING OF ESTROGEN IN THE FORMALIN-FIXED RAT UTERUS

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(*Received* 6 *June* 1989; *received for publication* 26 *April* 1990)

Summary—Rat uterus fixed overnight in buffered formalin retains the ability to specifically bind estradiol. However, the estrogen binding property of fixed tissue appears preferentially localized in the nuclear fraction regardless of hormonal status. Furthermore, the quantity of the nuclear estrogen receptor in fresh or fixed uterus is virtually identical in the presence or absence of estrogenic hormone. Yet, while both tissue preparations exhibit equivalent increases in the total nuclear receptor occupancy after hormone exposure, only the fresh uterus contains a major cytosolic estrogen binder which decreases in availability upon the estrogen-induced elevation of the nuclear bound steroid. However, the cytosolic estrogen receptor exhibits a significant loss in its ligand binding property after formalin exposure. Thus, the preferential localization of estrogen binding in the nuclear fraction of fixed whole tissue may just reflect that only the tightly bound nuclear estrogen receptor's functional and/or structural integrity survives long-term formalin fixation. Our observation of estrogen binding in preserved tissue may also be a clinically useful tool in therapy analysis.

INTRODUCTION

Estrogen binding is primarily localized to the cytoplasmic fraction (cytosol) of reproductive tissue homogenates in the absence of previous tissue or animal exposure to agonistic hormone. However, specific estrogen binding sites become localized predominantly to the nucleus after the tissue is exposed to estrogenic hormone [1–3]. Additionally, the cytosolic/nuclear distribution of estrogen after hormone treatment is consistent with a two-step mechanism of initial cytoplasmic hormone binding followed by nuclear translocation of the receptor–estrogen complex [4, 5]. This pattern of estrogen-dependent nuclear localization of hormone bining sites has also been observed *in vivo* during the rat estrous cycle [6].

More recent investigations have raised questions about the classical two-step mechanism of estrogen action and the principal cellular site of the estrogen receptor protein. The application of estrophilin antibodies to the study of the cellular dynamics of the estrogen receptor has suggested a nuclear presence for the steroid hormone receptor regardless of the physiological state of the tissue [7–9]. Cytochalasin B enucleation experiments have also indicated the presence of unoccupied estrogen receptor within the cell nucleus [10, 11]. There are also other reports in the literature which suggest that the experimental conditions used to prepare the tissue for subsequent estrogen binding assays can affect the cellular partitioning of the unoccupied receptor protein [12–14]. In this regard, it is interesting to note that the presence, absence and intensity of nuclear staining in the immunoperoxidase identification of estrogen receptor in human breast tumor was significantly associated with the concentration of cytosolic estrogen receptor determined by binding assay [9]. However, some technical questions have been raised which potentially confound the interpretation of the results for both the use of antibody and cytoplast enucleation techniques to elucidate the cellular localization of uncharged receptor [15, 16]. Indeed, results of immunocytochemical analyses of the cellular distribution of steroid receptors are not unequivocal [17, 18].

Most of the previous studies suggesting a nuclear presence for the estrogen receptor protein used cultured and/or tumor cell lines or biopsy material. In this report, we chose the direct approach of using the freshly obtained rat uterus as had been used in the initial elaboration of the cellular dynamics and mechanism of estrogen action. Since the structural conformation of the estrogen receptor in fixed cells is not so grossly altered as to obviate antibody recognition in the immunocytochemical localization studies, we rationalized that perhaps the integrity of the receptor's ligand binding site might also be preserved in fixed tissue. Consequently, we undertook experiments to determine the cellular localization of estrogen binding sites in fresh- and formalin-fixed uterine tissue in the absence or presence of estrogenic hormone.

EXPERIMENTAL

Sprague–Dawley rats (150–200 g) were bilaterally ovariectomized under ether anesthesia and uteri were obtained 2–4 weeks later for estrogen receptor assay. The rats were injected with 1 μg estradiol/100 g body wt 36 h prior to obtaining the uterine tissue to stimulate receptor production [19]. The uteri were quickly excised from cervically disarticulated rats, stripped of adhering connective tissue, finely minced with micro-dissecting scissors and placed into buffered (pH 7.4) calcium-magnesium-free Hanks' balanced salt solution containing 25 mM HEPES, 1 mM EDTA and 10% formalin (CMFH–formalin). The minced uterus was fixed overnight at 4°C in capped vials of CMFH-formalin. The next morning the tissue was subjected

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to alcoholic dehydration (30, 50, 70, 80, 95, 100, 100%) for 30 min at each step in the alcohol series. The uterine fragments were then homogenized on ice in CMFH solution using a motor-driven Kontes–Duall homogenizer. Fresh, minced uterine tissue was also processed in CMFH solution at the same time for concomitant estrogen receptor assay.

The tissue homogenates were centrifuged at 800 *g* at 4°C for 15 min. The charcoal [20] and nuclear exchange [21] assays were then utilized to measure the receptor population in the cytosolic and nuclear fractions, respectively. The crude cytosol (20 mg tissue equivalent) was incubated for 1 h at room temperature (22–23°C) with 18 nM 6,7 [^{3}H]estradiol (Amersham; SA 43 Ci/mmol) with or without 100-fold excess diethylstilbestrol (DES). The uterine nuclear fraction was washed two times with CMFH, resuspended at 20 mg tissue equivalent and incubated at 37°C for 30 min with 18 nM labeled estradiol plus or minus 100-fold excess competitor. After charcoal stripping the cytosol incubates and washing the nuclear incubates three times, aliquots of the cytosolic fraction and ethanol extracts of the nuclear fraction were counted in a Beckman LSC 100 scintillation counter to determine the quantity of specific estrogen binding.

RESULTS

As shown in Fig. 1, the estrogen receptor of fresh uterine tissue has a predominant cytosolic presence in the absence of hormone. However, 1 h after injection of 0.1 or 1.0 μg estradiol/100 g body wt there is a progressive decline in the availability of the cytosolic estrogen binding sites and an increasing nuclear occupancy of the bound steroid hormone. While this later observation is consistent with the classical two-step mechanism of estrogen action [2], it is in stark contrast to the distribution of estrogen binding sites in formalin-fixed uterine tissue. Although the estrogen receptor in fixed uterine tissue still retains the ability to recognize and specifically bind its ligand, the cellular distribution of the receptor appears preferentially localized to the nuclear fraction and is independent of the hormonal state of the tissue. Cytosolic binding of hormone is negligible. However, more interestingly, the nuclear availability of a specific estrogen binder in fixed tissue is essentially identical to the quantites of receptor present in fresh uterine tissue both before and after hormone exposure. This aspect is in contrast to the behavior of a number of the monoclonal antibody preparations against the estrogen receptor which stain target cell nuclei in the absence of hormone but fail to exhibit noticeable increases in nuclear staining intensity after estradiol treatment as expected [22].

In another experiment to assess the effect of formalin upon the integrity of cytosolic estrogen receptor binding, sufficient formalin was added to a high speed cytosolic uterine fraction to give a final concentration of 10% fixative. This was then incubated overnight at 4°C. The control cytosol pool had an equal volume of dilute HCl added to adjust for the slight pH change due to the added formalin. Determination of specific estrogen binding by the

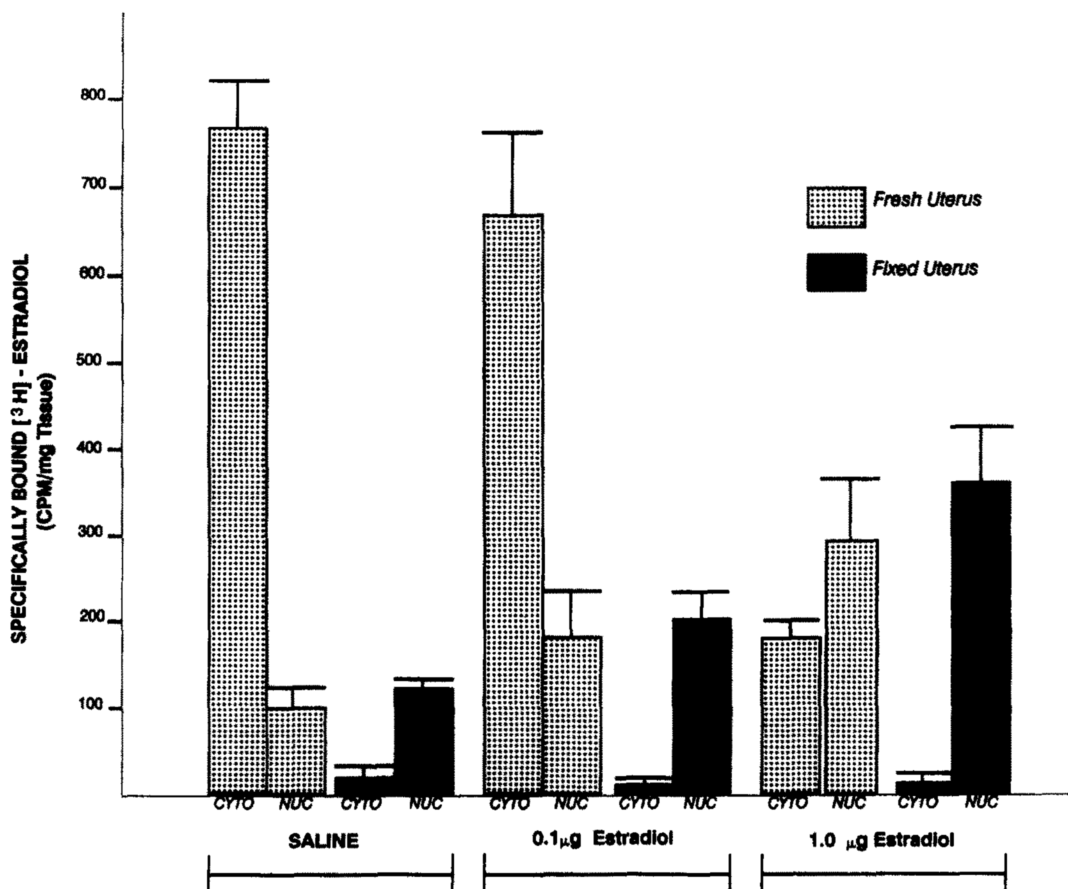

Fig. 1. Cytoplasmic and nuclear estrogen binding activity in fresh and formalin-fixed uterine tissue. Rats were injected s.c. with saline, 0.1 μg estradiol/100 g body wt or 1.0 μg estradiol/100 g body wt 1 h prior to obtaining the uterus for assay (fresh) or formalin fixation. The stippled bars represent estrogen binding in fresh uterus; the shaded bars, fixed uterus. Each bar graph represents the mean value of 3 (fresh) or 5 (fixed) single point binding assays. The SEM is indicated by the line above each bar.

charcoal–dextran assay [20] the next morning showed a 75% reduction in binding activity in the uterine cytosol aliquot exposed to formalin.

DISCUSSION

The observations we have made in this study raise questions about the nature of the cytosolic estrogen binder. Its presence in fresh tissue and virtual absence in fixed tissue might suggest that it reacts differently with the fixative solution than does the nuclear estrogen binder. To some extent this may be true, for when an estrogen receptor rich cytosolic fraction is exposed to the formalin fixative under the same conditions as whole uterine tissue, a significant reduction in measured estrogen binding capacity is observed. However, there still remains a moderate level (25% of total) of specific estrogen binding in the cytosol which is not seen in the formalin-fixed tissue, even though the protocol of the former determination requires measurement of hormone binding in the continued presence of the fixative. The possibility that unoccupied receptor becomes associated with cytoplasmic elements in the fixed tissue in such a way to preclude ligand binding, though, cannot be ruled out. Yet, the fact that both fresh and fixed tissue have the same quantity of nuclear estrogen binding might also seem to suggest that the presence of cytosolic receptor in fresh tissue may not be due to its artifactual extraction from the nucleus during tissue homogenization [12]. However, it is also possible that all estrogen receptor protein is initially in the nucleus with some molecules being more intimately associated with chromatin than others. The more loosely bound nuclear estrogen receptor may not survive formalin fixation similar to the situation when naked cytosolic receptor is directly exposed to formalin. Such a paradigm could also account for an increased nuclear presence of the estrogen receptor in the fixed tissue after prior steroid exposure due to a hormonally-induced stabilization of the receptor's nuclear binding (cf. [1–6, 12]). While there is a progressive decline in the availability of cytosolic binding sites in fresh tissue after exogeneous estradiol treatment, the increased nuclear occupancy of the receptor–estrogen complex is not commensurate with the observed decreases in the cytoplasmic binder. Thus, the relationship between the cytosolic and nuclear binding components is not entirely clear. That is, are the two components separate or derived entities? It is interesting to note that a pattern of hormonal binding for a class of potent uterotrophic estrogens has recently been observed which implicity suggests a functional disparity between the classical cytosolic and nuclear estrogen binders [23].

Thus, we report that fixed uterine tissue still retains the ability to specifically bind its ligand. This feature may be a useful adjunct to current procedures in the clinical assessment of therapeutic responsiveness and prognosis. Similarly, the hormone binding property of the formalin-fixed uterus is restricted to the cell nucleus as has been reported to be the case for a number of immunocytochemical studies [7–9]. This observation may be an indirect consequence of a differential susceptibility of cytosolic and nuclear receptor forms to fixative perturbation or the direct consequence of a restricted nuclear presence for the estrogen receptor.

Acknowledgements—We thank Dr James H. Clark for his review and discussion of the manuscript. This work was supported by National Science Foundation Research Grant PCM-8409586

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