J. Steroid Biochem. Molec. Biol. Vol. 43, No. 6, pp. 499–505, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

ESTROGEN RECEPTOR AND C_{19}-5-ENE-STEROID CONCENTRATIONS IN THE NUCLEAR FRACTION FROM HUMAN BREAST CARCINOMA TISSUE

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(Received 30 December 1991)

Summary—The adrenal-derived estrogen 5-androstene-3β,17β-diol (ADIOL) is estrogenic at the concentrations found in the blood of Western women. We have now measured the concentrations of both ADIOL and the estrogen receptor (ER) in the nuclear fraction (800 *g* pellet) of 89 primary human mammary tumors. No difference was found in nuclear ADIOL concentrations in tumors from 45 pre- and 44 postmenopausal women. Significantly higher nuclear ADIOL concentrations were found in 49 ER negative tumors compared to 40 ER positive tumors ($P < 0.005$). A similar relationship applied in the postmenopausal group ($P = 0.01$) and the premenopausal group, but in this latter instance failed to reach significance ($P = 0.1$). In ER positive tumors there was no correlation between ADIOL and ER nuclear levels. ADIOL was present in the total particulate fraction (100,000 *g* pellet) at twice the concentration found in the nuclear 800 *g* pellet and again no difference was found in its concentration in tumors from 20 pre- compared to 34 postmenopausal women. Dehydroepiandrosterone was also measured in the 800 *g* fraction of 45 tumors and its concentration, which was some 10-fold higher than ADIOL and significantly correlated with that steroid, was again independent of menopausal status. The higher concentration of C_{19}-5-ene-steroids in ER negative cellular fractions could be due to differences in their metabolism; ER negative tumors either lack, or possess very low levels of, hydroxysteroid sulfotransferase which catalyzes formation of sulfate esters of C_{19}-5-ene-steroids previously observed to be major metabolites produced by ER positive cells. Higher concentrations of free steroids in ER negative cells would then be available for combination with membranes and non-specific binding sites throughout the cell.

INTRODUCTION

Estrogen action, via ligand binding to estrogen receptor (ER), is not confined to the classical estrogens estradiol-17β (E_2), estrone, and estriol. Androgens, for example, if present in sufficiently high concentration to combine with ER, act as estrogen agonists in some systems [1–3]. 5-Androstene-3β,17β-diol (ADIOL) which is still classified as belonging to the so-called "adrenal androgen" group, was demonstrated to be an estrogen by Charles Huggins and his colleagues in 1954 [4]. This steroid was later found to possess high affinity for ER ($K_d \sim 5$nM) [5–7] and to act as an estrogen at concentrations found in the blood of Western women. This topic was the subject of a review in 1983 [8] and since that time many additional studies have confirmed that ADIOL is estrogenic in a number of model systems [9–13]. This estrogenic action occurs without aromatization [9].

Interest has focused on ADIOL because of its possible involvement in the etiology of breast cancer [8]. Its concentration in the blood is maintained by a "buffer" type mechanism involving its precursor dehydroepiandrosterone sulfate (DHEAS) [14]. In addition, certain tissues, including human breast cancer [15], have the ability to cleave DHEAS and thus provide an additional source of ADIOL by 17β-hydroxysteroid dehydrogenase action. Higher concentrations of DHEAS occur in the blood of Western versus Japanese women [8], and resulting elevated ADIOL levels may provide a constant estrogenic stimulus, which in postmenopausal women would be unopposed by progesterone.

It is now realised that the concentrations of steroid hormones in the blood do not necessarily reflect concentrations found in tissues. A new term "Intracrinology" has been introduced

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to describe those transformations and processes responsible for this phenomenon [16]. In a previous communication, we reported the presence of ADIOL in the cytosol prepared from primary human mammary tumors. We aimed to establish whether any correlation existed between its concentration and the concentration of ER in cytosols. No such correlation was found [17]. We have now focused attention on the nuclear fraction which would be expected to contain ligand-charged ER. Nuclear ER (ER_N) concentrations have been determined in the washed 800 *g* fraction from human breast tumors by an exchange assay. No correlation was found between ER_N and ADIOL or DHEA concentrations in this nuclear fraction. Higher levels of these steroids were found in nuclei from tumors lacking ER and this could be due to the inability of such tumors to metabolize C_{19}-5-ene-steroids to water-soluble sulfate conjugates.

EXPERIMENTAL

Materials

Chemicals and radiochemicals were described previously [17]. The ADIOL antisera was kindly supplied by Dr John Moore, (Imperial Cancer Research Fund, London, England). The highly specific DHEA antisera was purchased from Sigma (St Louis, MO). Human mammary carcinoma tissue was delivered to the laboratory in dry ice and subsequently stored at −70°C for a period not greater than 3 weeks. Thawed tissue was dissected free of fat and connective tissue and after mincing was frozen in liquid N_2 and pulverized for 2 min in a microdismembranator (Mickro-dismembranator II, Braun, Melsunger, Germany). The powdered material was transferred to polypropylene tubes and homogenized in 4 vol of 10 mM Tris–HCl, pH 7.4, containing 1.5 mM EDTA and 1 mM dithiothreitol (TED buffer), using an Ultraturrax instrument (Janke and Kunkel, KG Ikawerk, Stausen I. Breisgaw, Germany).

Pellets (800 g)

Homogenates, prepared from tumor samples as described above, were centrifuged at 800 *g* for 10 min. The supernatants were then centrifuged at 100,000 *g* for 1 h and ER concentrations determined on the cytosol as described previously [17]. ER values above 6 fmol/mg protein were regarded as ER positive. The 800 *g* pellets were washed twice by resuspending in 15 ml TED buffer, pH 7.4, and centrifuging at 800 *g*. Washed pellets were then suspended in 10 ml TED buffer, pH 7.4, and centrifuged at 100,000 *g*. This final centrifugation was carried out to stabilize ER_N by removal of proteases present in the soluble fraction [18].

Pellets (100,000 g)

In this case, homogenates prepared as above, were centrifuged directly for 1 h at 100,000 *g*. Pellets were resuspended in 10 ml of TED buffer, using a loosely fitting glass–Teflon homogenizer. This was then centrifuged at 100,000 *g* for 20 min and the process of resuspension and centrifuging repeated. The final pellet was suspended for assay of ADIOL and ER_N as described in the next sections.

ER_N determination

The final washed nuclear and 100,000 *g* fractions obtained as described in the above procedures were suspended with the aid of a glass–Teflon homogenizer in 3 ml TED buffer, pH 8.5, containing 0.5 M KCl. After standing for 30 min at 0°C, the suspension was divided into two portions. One portion was stored at −20°C for RIA of ADIOL and DHEA and the remaining portion centrifuged at 100,000 *g* for ER_N determination on the extract. Pellets were kept for DNA analyses. Duplicate aliquots (200 μl) of the 0.5 M KCl extracts were incubated at 25°C for 15 h with 13 nM [^{3}H]E_2 ± 1.3 μM diethylstilbestrol in a total volume of 300 μl. After cooling in ice, 100 μl of dextran-coated charcoal (2.0% Norit A charcoal and 0.05% dextran in TED buffer, pH 7.4) was added and after 20 min the tubes were centrifuged and aliquots of supernatant taken for liquid scintillation counting in a Packard 2660 instrument equipped with external quench correction. Geier *et al.* [18] had demonstrated that the ER_N, extractable into KCl from washed 100,000 *g* pellets from human mammary tumors, was stable for at least 6 h at 30°C. Four ER_N positive tumors were assayed for ER_N content after 6 and 16 h incubation with [^{3}H]E_2 at 25°C. Identical results were obtained in each case and the 16 h time was routinely adopted. Values of ER_N above 60 fmol/mg DNA were regarded as ER_N positive.

RIA of ADIOL and DHEA

The thawed 0.5 M KCl suspensions were sonicated for 5 s to disperse gelatinous material and after addition of an equal volume of water

they were extracted 3 times with 5 ml volumes of ether. These extracts were then dried and stored. The RIA methods employed for ADIOL and DHEA determinations in subcellular breast tumor preparations have been described previously [17]. Although the antisera used for ADIOL was very specific [19], determinations on plasma extracts required a purification step due to the presence interfering substances [19]. However, we found that purification of ADIOL and DHEA from breast tumor subcellular fractions, prior to assay with their respective antisera, was unnecessary—values within 12% were obtained for both ADIOL and DHEA with and without separation by TLC. Also of importance was the fact that DHEA, when present in amounts ranging from 20 to 10^4 pg/assay tube, was without effect on the assay of ADIOL. These, and other details of the assessment of the RIA methods, are given in Ref. [17].

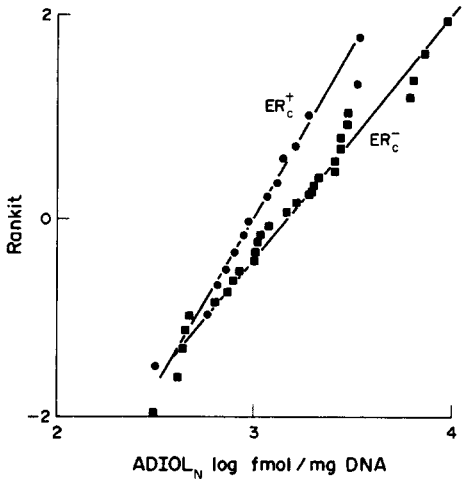

Fig. 1. Rankit test demonstration (see text) of logarithmic distribution of ADIOL in the 800 *g* nuclear fraction of tumors from premenopausal women. The higher concentration found in ER negative tumors did not reach statistical significance (see Table 1). ER_C^+ and ER_C^- refer to ER status determined in tumor cytosols.

Other methods

Protein was determined by the method of Lowry *et al.* [20] and DNA by the method of Burton [21]. Statistical significance between C_{19}-5-ene-steroid concentrations were determined on log transformed values employing Student's *t*-test.

RESULTS

ADIOL and ER_N determinations in the 800 g nuclear fraction

Normality of frequency distribution of ADIOL values in the 800 *g* nuclear fraction was examined graphically by plotting values against ranked normal deviates, or rankits, obtained from statistical tables [22]. A straight line relationship only applied when values were log transformed. Results for ER positive and negative tumors from premenopausal subjects are shown in Fig. 1, and for postmenopausal subjects in Fig. 2. Brownsey *et al.* [23] have reported that plasma levels of DHEAS are also logarithmically distributed. An unexpected finding was the higher ADIOL concentration found in ER negative tumors in both groups. However, this reached significance for postmenopausal subjects only (Table 1). When the collective data was examined without regard to menopausal status, ADIOL concentrations in the nuclear fraction were highly significantly elevated in ER negative tumors ($P < 0.005$, Fig. 3).

ER_N values were also found not to be distributed normally, but fitted a normal distribution when log transformed, as has been previously reported [24]. For ER positive tumors, there was no significant correlation between ER_N and ADIOL concentrations ($r = 0.1$).

In contrast to the higher blood concentrations of C_{19}-5-ene-steroids, including ADIOL, in premenopausal women [8], there was no difference in the ADIOL levels in the nuclear fraction from tumors obtained from pre- and postmenopausal subjects (Table 1).

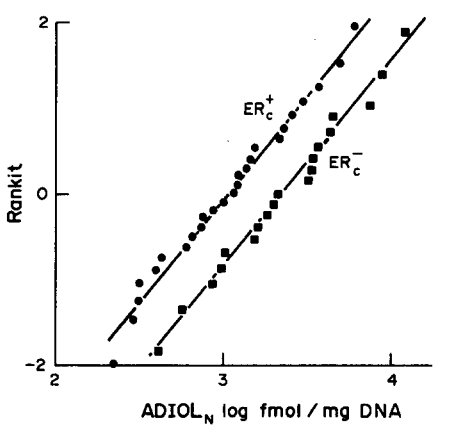

Fig. 2. Rankit test demonstration of logarithmic distribution of ADIOL in the 800 *g* nuclear fraction of tumors from postmenopausal women. Significantly higher concentrations were present in ER negative tumors ($P = 0.01$, see Table 1).

Table 1. ADIOL and ER_N concentrations (mean ± SD) in 800 *g* nuclear fractions from 89 primary human mammary tumors

	ER positive (+)[a]		ER negative (−)	*P*
Menopausal status	ADIOL (log fmol/mg DNA)	ER_N	ADIOL (log fmol/mg DNA)	ADIOL ER + vs ER −
Pre	3.02 ± 0.31	2.4 ± 1.05 (n = 15)	3.19 ± 0.39 (n = 30)	0.1
Post	3.00 ± 0.37	1.95 ± 1.42 (n = 25)	3.36 ± 0.39 (n = 19)	0.01

[a]Mean ± SD concentrations of ER (fmol/mg protein) determined in cytosols were: premenopausal, 56 ± 76; postmenopausal, 89 ± 105.

In 6 tumours ADIOL levels were below the sensitivity of the method and have not been considered. The minimum detectable dose was determined by applying 95% confidence limits to the zero dose of the standard curve for each assay. This sensitivity was usually 10 pg.

There was no significant difference in ADIOL levels in the pre- and postmenopausal groups.

ADIOL concentrations in 100,000 g pellets

In order to obtain additional information about the subcellular distribution of ADIOL in breast tumors, assays were performed on the total particulate fraction obtained by centrifuging homogenized tumor tissue (prepared in an identical manner to the isolation of the 800 *g* fraction) at 100,000 *g*. Results from 54 tumors (20 pre- and 34 postmenopausal), expressed as ng/g wet weight, are compared with the previous data on the 800 *g* nuclear fraction in Table 2. It can be seen that the concentration of ADIOL in the total particulate fraction is about double that in the 800 *g* nuclear fraction. Thus the steroid is distributed in microsomes and mitochondria, nuclei, and cytosol [17].

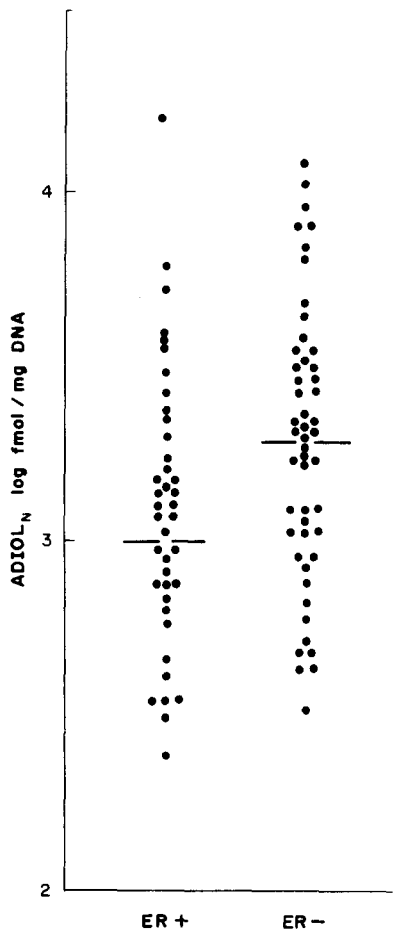

Fig. 3. ADIOL concentrations in 800 *g* nuclear fraction of 89 mammary tumors with respect to ER status. Higher concentrations were found in ER negative vs ER positive tumors ($P < 0.005$).

DHEA concentrations in the 800 g nuclear fraction

In 45 tumors DHEA was measured in this fraction together with ADIOL. DHEA concentrations were some 10-fold higher than ADIOL, and although also higher in ER negative compared to ER positive tumors from both pre- and postmenopausal subjects, this difference was not significant. As had been established for ADIOL, there was no difference in the concentration of DHEA in the latter groups (Table 3).

Correlation of ADIOL and DHEA levels

Figure 4 shows that ADIOL and DHEA concentrations in the 800 *g* fraction were correlated ($P < 0.001$). When this relationship was separately examined in pre- and postmenopausal subjects [Fig. 4(A)], there was no significant difference in slopes of the regression lines. In a similar fashion, there was no significant difference in slopes of the regression lines for ER positive and negative tumors [Fig. 4(B)]. This makes it unlikely that the difference in ADIOL concentrations between ER positive

Table 2. ADIOL distribution in the 800 *g* nuclear fraction and the total particulate fraction of human mammary tumors

Fraction	Pre [ng/g wet weight (mean ± SD)]	Post
800 *g* pellet	0.41 ± 0.23 (n = 45)	0.52 ± 0.44 (n = 44)
100,000 *g* pellet	0.93 ± 0.91 (n = 20)	0.99 ± 0.90 (n = 34)

Table 3. DHEA concentrations (means ± SD) in the 800 *g* nuclear fraction from 45 primary human mammary tumors

Menopausal status	ER +	ER −
	(log fmol/mg DNA)	
Pre ($n = 23$)	3.77 ± 0.50	3.94 ± 0.42
Post ($n = 22$)	3.99 ± 0.32	4.06 ± 0.47

There were no significant differences with respect to menopausal status. The higher concentrations of DHEA in ER − tumors did not reach significance.

and negative tumors is due to differences in 17β-hydroxysteroid dehydrogenase levels.

DISCUSSION

DHEAS is a major secretory product of the adrenal cortex. DHEA and ADIOL in the blood are mainly derived from this secreted DHEAS; the concentrations of all three steroids being highly correlated with one another [14, 25]. C_{19}-5-ene-steroid concentrations in human breast tumor tissue have been determined by a number of workers. Mean concentrations of DHEA in two studies were both of the order of 16 ng/g wet weight, with values ranging from 1.6–160 ng/g [26], and 3.3–65 ng/g [27]. A third study reported a range of values from 2.5–466 ng/g wet weight [28], and all three studies found that DHEA concentrations were significantly correlated with those of ADIOL–DHEA being present in a 10- to 20-fold higher concentration. This was also the situation in our previous study on breast tumor cytosols [17], and is again evident in the current data on the nuclear fraction (Table 3, Fig. 4). When one considers that DHEA and ADIOL plasma levels are 4.5 and 0.75 ng/ml, respectively, in premenopausal women, and 1.5 and 0.33 ng/ml, respectively, in postmenopausal women [29, 30], a considerable tissue–plasma gradient has occurred. Although DHEAS plasma concentrations differ in pre- and postmenopausal Western women, nevertheless in both instances the levels are exceedingly high (2 and 0.5 μg/ml, respectively [29]). This could possibly account for ADIOL and DHEA concentrations in subcellular fractions of breast tumors being independent of menopausal status—if plasma DHEAS was their major precursor and sulfatase action was implicated.

Despite an overlap in individual values, a higher concentration of ADIOL was found in the 800 *g* fraction of ER negative tumors which is in keeping with the general results of Maynard *et al.* [28], determined on human mammary tumor specimens by GLC/mass spectrometry. Their ADIOL values were similar to our own, if levels in tumor cytosols [17] are added to those found in the 100,000 *g* pellets (Table 2). In the study of Maynard *et al.*, no overall correlation was found between DHEA or ADIOL levels with those of ER in the cytosol. However, 7 out of 10 tumors with high DHEA (> 100 ng/g), and 5 out of 6 tumors with high ADIOL (> 20 ng/g), were all ER negative. Since intracellular concentrations of ADIOL are not positively related to ER_N—indeed higher concentrations being found in tumors lacking ER—one must consider the presence of a separate specific receptor for ADIOL and/or DHEA, or invoke differences in metabolism of C_{19}-5-ene-steroids in ER positive and negative mammary tumor cells. Although the presence of a specific receptor for ADIOL in rat vagina has been claimed [31], this has not been substantiated, and all the evidence is consistent with ADIOL acting via ER to initiate biological responses. Recent studies on C_{19}-5-ene-steroid metabolism

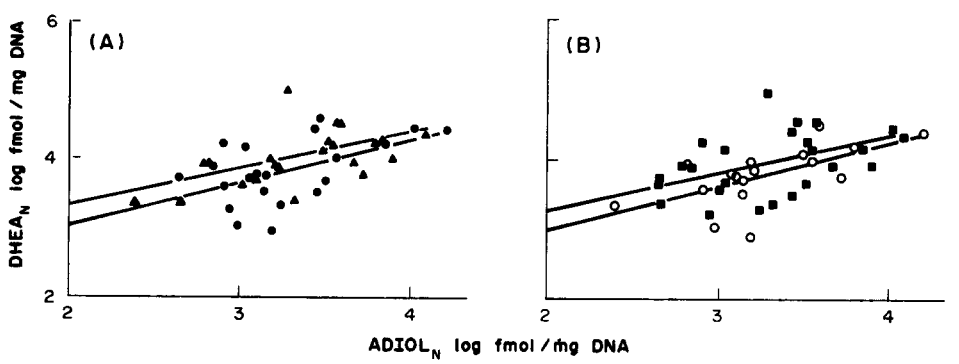

Fig. 4. Correlation between ADIOL and DHEA concentrations in the 800 *g* nuclear fractions. (A) Data shown for pre- (●) and postmenopausal (▲) women. (B) Data shown for ER positive (○) and negative (■) tumors. There was no significant difference in the regression lines for post- (top line) and premenopausal (bottom line) subjects in (A), or ER negative (top line) and positive (bottom line) tumors in (B). For all samples, $r = 0.50$, $P < 0.001$.

by human mammary cancer tissue and cell lines in culture, do show however that expression of enzymes which metabolize these steroids is related to expression of ER. For example, ER positive human mammary tumors contain higher levels of hydroxysteroid sulfotransferase (the enzyme which sulfurylates ADIOL and DHEA) than ER negative tumors [32]. Furthermore, whilst 4 ER positive human mammary cancer cell lines formed sulfate esters of ADIOL as major products of metabolism, only 1 of 4 ER negative cell lines sulfurylated ADIOL, and this was at a rate 12-fold lower compared to the mean rate for the ER positive cell lines [32]. In addition, both ER positive and negative human mammary cancer cell lines accumulate ADIOL and DHEA within the cell as long chain fatty acid esters. However, a much more rapid rate of accumulation occurs in ER negative cells [33, 34]. These esters can be reconverted to the free steroid and thus provide a means of concentrating the hormones within the cell [33, 34]. Such differences could also favor higher concentrations of C_{19}-5-ene-steroids in ER negative tumors.

We conclude that no differences in ADIOL concentrations occur in subcellular fractions of breast tumors from pre- and postmenopausal women, despite differences in plasma concentrations. These intracellular concentrations would be sufficiently high to combine with ER, although no correlation was found between ER_N and ADIOL concentrations in the nuclear fraction. The higher concentrations of ADIOL found in ER negative tumors, are very likely due to the absence (or diminished levels) of hydroxysteroid sulfotransferase; an enzyme which in ER positive human mammary cancer cells is responsible for eliminating C_{19}-5-ene-steroids as polar sulfate esters.

Acknowledgements—This work was supported by a grant from the National Health and Medical Research Council of Australia. We wish to thank Mr A. Kennerson and Ms K. P. Ho for carrying out the estrogen receptor assays in cytosols.

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