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REDUCTION OF PROLINE TRANSPORT IN R3230AC MAMMARY CARCINOMAS BY ESTROGENS *IN VITRO**

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Abstract—The effects of estrogens on proline and leucine transport into enzymatically dissociated cells from the R3230AC mammary adenocarcinoma were studied. Estradiol-17β demonstrated a time- and dose-dependent reduction of proline transport; at 10^{-6} M, transport of proline was decreased by 50 percent. Kinetic analysis of these effects indicate that estradiol displayed characteristics of a non-competitive inhibitor, with a K_i of 1.79 μM. Other estrogens, and the anti-estrogen tamoxifen, gave somewhat higher estimated K_i values and could be ranked as inhibitors as follows: estradiol-17β > diethylstilbestrol > tamoxifen > estriol ≃ estrone > estradiol-17α. No effects of these estrogens on leucine transport were observed, indicating their selectiveness for the A system. The synthetic glucocorticoid, dexamethasone, was comparable to diethylstilbestrol as an inhibitor of proline transport, but dexamethasone also decreased transport of leucine. Testosterone and progesterone were approximately comparable to estrone in their actions. It is proposed that these actions of estrogens represent one potential mechanism whereby pharmacological levels exert therapeutic benefit in the treatment of advanced breast cancer.

In a recent review, Guidotti *et al.* [1] noted that estrogens affect amino acid transport in target tissues known to possess receptors, such as the uterus. In these studies *in vivo* and *in vitro*, the uptake of α-aminoisobutyric acid (AIB) was increased after hormonal treatment and kinetic analysis of this response revealed that the enhanced entry was accountable to increases in V_{max} with little or no effect on K_m [2, 3]. The characteristics of this transport response support the conclusion that estrogens affected entry via the A system [4, 5]. In contrast to trophic effects of estrogens, little is known about their actions to inhibit growth or cause regression, such as in mammary tumors.

Studies in our laboratory were conducted with the R3230AC mammary adenocarcinoma, a transplantable hormone-responsive neoplasm [6], to ascertain whether changes in substrate transport were correlated with alterations in tumor growth behavior resulting from hormonal manipulation of the host. After characterizing neutral amino acid transport systems in this tumor [7, 8], we reported that altered tumor growth was accompanied by appropriate changes in substrates transported by the A system [9, 10]. We observed that estrogen treatment produced a decrease in transport of proline, an effect compatible with reduction in tumor growth [11].

We then conducted experiments to examine in more detail the kinetic characteristics of this estradiol-induced decrease in transport. Additionally, specificity was investigated by assessing the effects of several steroidal estrogens, diethylstilbestrol, an anti-estrogen and representatives of other classes of steroid hormones. The data indicate that potent estrogens were the most effective inhibitors of the A system, displaying kinetic characteristics of non-competitive antagonists. We suggest that one possible mechanism of action of pharmacological doses of estrogens to inhibit tumor growth occurs by inhibition of amino acid transport.

MATERIALS AND METHODS

Female Fischer rats (80–90 g) were obtained from the Charles River Breeding Laboratory (Wilmington, MA). The R3230AC tumor was implanted subcutaneously in the axillary region on both sides by a sterile trochar technique as described by Hilf *et al.* [12].

Diabetes was induced by intravenous administration of streptozotocin 2 days prior to tumor implantation [13]. Streptozotocin was dissolved in 0.9% NaCl and rapidly adjusted to pH 4.5 with 0.025 M citric acid. Injections of 70 mg/kg were made within 30 min. Animals were classified as diabetic based on blood glucose exceeding 250 mg/100 ml, and urinary glucose, estimated by Clinistix (Ames Co., Inc., Elkhart, IN), greater than 0.5 g/100 ml. Serum insulin levels were $<10^{-10}$ M as measured by radioimmunoassay. Since tumors from diabetic rats demonstrated enhanced proline transport [8–10], it was decided to conduct all experiments on cells from diabetic animals.

Animals were killed by cervical dislocation 2.5 to 3.5 weeks after tumor implantation. Tumors were quickly excised and placed in ice-cold Hanks' balanced salt solution (without calcium and magnesium). Generally, tumors from three to five rats were used. After removal of connective and necrotic tissues, approximately 3 g of tumor tissue was minced into 1 × 1 mm pieces on a McIlwain tissue slicer

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(Brinkmann Instruments, Inc., Westbury, NY). The minced tissue was incubated with 10 ml of Hanks' balanced salt solution containing 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) and 0.05% collagenase (Type II, Worthington Biochemical Corp., Freehold, NJ) for 15 min at 37° in a Benco shaking water bath (approximately 50 cycles/min). The tissue was strained through a 100 mesh stainless steel strainer and incubated with fresh enzyme solution for another 60 min. Details of this procedure were published previously [14]. Cell viability was estimated by trypan blue exclusion, and cell number was determined by the use of a hemocytometer. Cell preparations used had more than 85 percent viability.

Proline transport was measured on 5×10^6 cells in plastic Falcon tubes in a final volume of 1 ml in HEPES* buffer, pH 7.4, containing 25 mM HEPES, 10 mM $NaHCO_3$, 125 mM NaCl, 3 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 11 mM glucose with a final osmolarity of 310 mOsM. To measure entry not mediated by a carrier, Na^+ was omitted; NaCl and $NaHCO_3$ were replaced by choline chloride and choline bicarbonate at equimolar concentrations.

Initial velocity measurements were determined by incubating cells in triplicate with the appropriate concentrations of labeled and unlabeled proline for 20, 40 and 60 sec at 37° in a shaking water bath. Transport was terminated by the rapid addition of 8 ml of ice-cold 0.9% NaCl followed by centrifugation for 2.5 min at 900 *g*. The cells were washed with ice-cold 0.9% NaCl and centrifuged as before. The tubes were inverted to drain and the sides were wiped clear of remaining liquid. The cell pellet was dissolved in two successive 5-ml portions of Aqueous Counting Scintillant (Amersham/Searle, Arlington Heights, IL), and radioactivity was counted in an Isocap 300 liquid scintillation counter (Nuclear Chicago, Chicago, IL). The efficiency for counting ^{14}C was 60 percent and ^3H, 40 percent.

Although at low concentrations leucine enters almost exclusively by the L system, we nevertheless measured leucine transport in the presence of 50 mM proline, to prevent entry of leucine by the A system. To measure the non-mediated entry of leucine, uptake of labeled leucine was measured in cells incubated in a HEPES minus Na^+ buffer, containing 50 mM unlabeled leucine to saturate the carrier (K_m for L system was ~0.5 mM) [10]. Otherwise, leucine transport assays were performed in the same manner as for proline transport.

To determine alterations in proline transport by various hormones, the cells were initially incubated in the presence of the particular hormone for 1.5 hr. Most hormones were studied at concentrations of 10^{-6}, 5×10^{-7} and 10^{-7} M, except for estradiol-17β, where 10^{-8} and 10^{-10} M were also used. Proline transport was measured at substrate concentrations of 0.1, 0.5 and 2 mM. Leucine transport was measured at a substrate concentration of 0.1 mM, following the 1.5-hr incubation with the various hormones at a single concentration of 1×10^{-6} M.

Chemicals. HEPES, NaCl, choline chloride, choline bicarbonate, proline, leucine and dexamethasone were obtained from the Sigma Chemical Co. Estradiol-17α and testosterone were obtained from the Research Plus Steroid Laboratory, Denville, NJ. Estradiol-17β, estrone, estriol and progesterone were obtained from CalBiochem, La Jolla, CA. Diethylstilbestrol was obtained from the National Cancer Institute, Bethesda, MD. Tamoxifen was obtained from Stuart Pharmaceuticals, Wilmington, DE.

L-[U-^{14}C]Proline (sp. act. 283 mCi/mmole) and L-[4,5-^3H]leucine (sp. act. 46 Ci/mmole) were obtained from Amersham, Arlington Heights, IL.

RESULTS

Time-course of reduction of proline transport. Initial experiments were conducted to ascertain the time-course of reduction of proline transport by estradiol-17β. At 10^{-6} M estradiol, uptake of proline (0.1 mM) was gradually reduced (Fig. 1), with no apparent effects by 15 min but significant decreases observed at 30 min after addition of the steroid to the incubation medium. By 90 min proline transport was decreased by 50 percent, an effect that was not enhanced further by 120 min. Based on these findings, subsequent transport assays were conducted after 90 min incubation periods with hormones.

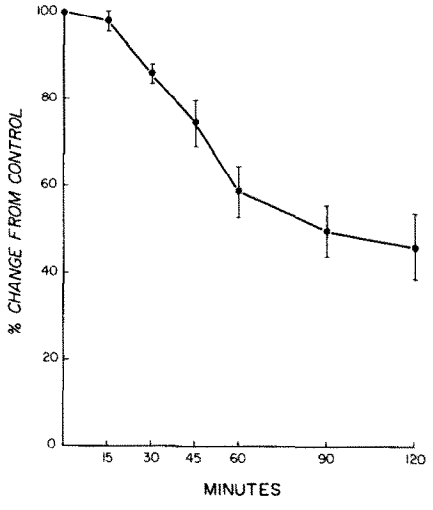

Fig. 1. Time-course of estradiol-17β (10^{-6} M) inhibition of 0.1 mM proline transport. Each point is an average of three separate experiments (mean ± S.E.M.) in which the initial velocity of transport (100 percent represents 39 ± 12 pmoles · min^{-1} · 10^6 $cells^{-1}$) was determined by linear regression analysis of data from triplicate samples at 20, 40 and 60 sec (correlation coeffcient greater than 96 percent).

Effects of estadiol-17β on proline transport. It was noted earlier [11] that estradiol-17β was capable of partially antagonizing the insulin-induced stimulation of proline transport as well as decreasing proline transport when the steroid was examined in the absence of insulin. We conducted experiments to characterize kinetically the effects of estradiol-17β on proline transport into dissociated cells of the R3230AC mammary carcinoma. Kinetic analysis of

* Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Table 1. Effects of dose of estradiol-17β on proline transport*

Level of estradiol-17β (M)	V_{max} (pmoles·min^{-1}·10^6 cells^{-1})	K_m (mM)	r
0 (control)	645	0.53	0.973
1×10^{-10}	610	0.53	0.976
1×10^{-8}	605	0.55	0.989
1×10^{-7}	565	0.56	0.997
5×10^{-7}	496	0.57	0.972
1×10^{-6}	381	0.54	0.995

* Proline transport was measured 90 min after incubation of tumor cells with various levels of estradiol-17β. Initial velocity of entry of proline, at 0.1, 0.5 and 2.0 mM, was determined from triplicate samples at 20, 40 and 60 sec. The data were analyzed by linear regression procedures. These data were then plotted as v_i vs $v_i/[S]$; linearity was assessed by goodness of fit (correlation coefficient = r).

the data was performed by linear regression techniques, plotting v_i vs $v_i/[S]$ (Woolf–Augustinsson–Hofstee plot); goodness of fit was calculated (r). Over the range of 10^{-10}–10^{-6} M estradiol-17β (Table 1), a progressive decrease in proline transport (V_{max}) was obtained, depicted as a series of parallel lines; K_m values for proline transport estimated from these data ranged from 0.52 to 0.56 mM. These results suggested that estradiol-17β acted as a non-competitive inhibitor of proline uptake, based on the effects seen for V_{max} with no effect on K_m. These data were analyzed according to the method of Dixon [15]. The results agree with the anticipated behavior of a non-competitive inhibitor, with the lines converging to a common intersection on the $[I]$ axis (Fig. 2). Since at this point $[I] = -K_i$, we estimated the K_i for estradiol-17β as being 1.8 μM.

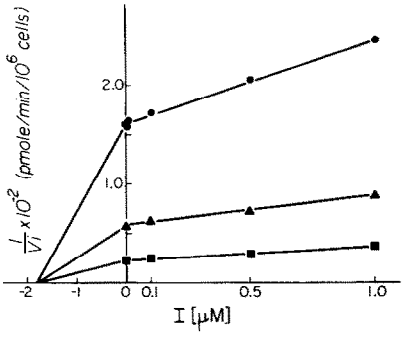

Fig. 2. Kinetic analysis of effects of various concentrations of estradiol-17β on proline transport depicted according to the method of Dixon [15]. Concentrations of proline used were: 0.1 mM (●), 0.5 mM (▲) or 2 mM (■). Lines shown and intercepts on axes were obtained by linear regression analysis; r was >0.98. Note that the scale on the negative $[I]$ axis was condensed due to considerations of space.

In 1974, Cornish-Bowden [16] suggested a simple graphical method for determining inhibition constants in which S/v_i can be plotted against $[I]$; the intersection provides an estimate of K_i' for uncompetitive, mixed or non-competitive inhibitors. When our data for estradiol-17β were plotted according to Cornish-Bowden, a common intersection on the $[I]$ axis was obtained (Fig. 3), the intercept being 1.8 μM. This similarity with the Dixon plot would be expected for a non-competitive inhibitor.

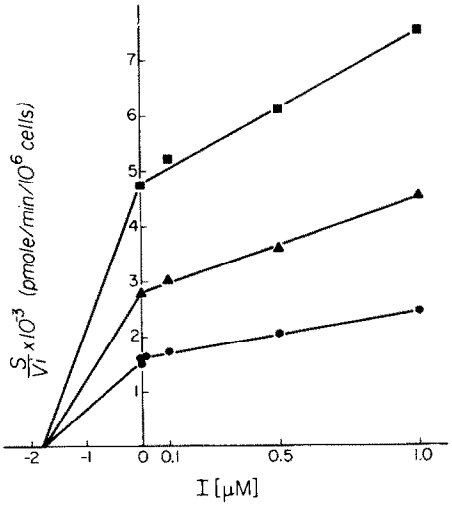

Fig. 3. Kinetic data from Fig. 2 plotted according to the method of Cornish-Bowden [16]. Concentrations of proline used were: 0.1 mM (●), 0.5 mM (▲) or 2 mM (■). Lines shown and intercepts on axes were obtained by linear regression analysis; r was >0.98. Note that the scale on the negative $[I]$ axis was condensed due to considerations of space.

Effects of estrogens and anti-estrogens on proline transport. It was of interest to examine the ability of other estrogens to reduce proline transport into cells of this tumor. Dixon plots were constructed from data obtained with three substrate concentrations of proline (0.1, 0.5 and 2.0 mM) and transport was assayed after exposure to various concentrations of estrogens (usually from 10^{-8} to 10^{-6} M). The data for v_i were subjected to linear regression analysis to determine the best fitting line; intercepts were calculated from this analysis. The data are summarized in Table 2, along with the results for estradiol-17β for comparison. These data indicate that estradiol-17β had the lowest K_i; diethylstilbestrol demonstrated a higher K_i value. For the naturally occurring steroidal estrogens, the relative order of potency based on the estimated K_i was estradiol-17β > estriol ≃ estrone, a ranking that resembled their potency as estrogens (based on bioassay). Estradiol-17α, the isomer of estradiol-17β (a rather weak estrogen by bioassay), gave a higher value of

K_i for inhibition of proline transport compared to those obtained for the more potent estrogens. The results observed with the anti-estrogen tamoxifen were surprising; the estimated K_i for inhibition of proline transport was lower than that calculated for estriol or estrone, which are more potent estrogens. To determine whether the anti-estrogen tamoxifen could antagonize the effects of estradiol-17β on proline transport, cells were exposed to a mixture of both compounds at equal concentrations. The resulting estimated K_i was approximately midway between the K_i values of each agent alone, implying some interactions between those sites responsible for the effects on transport. A similar effect was observed for a mixture of estradiol-17β and diethylstilbestrol.

We also examined the effects of representatives of other classes of steroids on proline transport (Table 2). Dexamethasone, a synthetic potent glucocorticoid, inhibited proline transport with an estimated K_i of 2.76×10^{-6} M. Progesterone and testosterone were also studied under these conditions; both gave values for the estimated K_i of $\sim 5.0 \times 10^{-6}$ M.

The data were also analyzed by the graphical method of Cornish-Bowden [16]. In general, for the estrogens, good agreement was obtained for the estimated K_i' with those K_i values obtained by the method of Dixon, although the K_i' tended to be slightly lower.

Specificity of inhibition on transport. Proline enters these cells by the A system, whereas leucine entry, particularly at low concentrations, is indicative of the L system [7, 8]. We examined the effects of these hormonal agents on both proline and leucine transport in preparations of cells from the same tumors under similar conditions. The data, summarized in Table 3, demonstrate that none of the estrogens nor the anti-estrogen tamoxifen had a significant effect on leucine transport at the same concentrations at which they displayed a significant effect on proline transport. Testosterone and progesterone demonstrated little difference in their abilities to decrease entry of proline and leucine, implying less specificity to their action on amino acid transport. However, dexamethasone showed a modest but significant effect on transport of leucine.

Table 2. Estimated inhibition constants of hormones for proline transport in R3230AC mammary tumor cells*

Hormonal agent	K_i ($\times 10^{-6}$ M)	K_i' ($\times 10^{-6}$ M)
Estradiol-17β	1.79 ± 0.03	1.79 ± 0.02
Diethylstilbestrol	3.06 ± 0.09	2.47 ± 0.09
Tamoxifen	4.11 ± 0.16	4.30 ± 0.23
Estriol	4.74 ± 0.11	3.44 ± 0.04
Estrone	5.46 ± 0.07	4.64 ± 0.10
Estradiol-17α	6.93 ± 0.30	5.56 ± 0.06
Estradiol-17β + diethylstilbestrol	2.55 ± 0.15	2.61 ± 0.19
Estradiol-17β + tamoxifen	3.30 ± 0.03	3.62 ± 0.08
Dexamethasone	2.76 ± 0.04	1.26 ± 0.01
Progesterone	5.03 ± 0.01	5.10 ± 0.01
Testosterone	4.92 ± 0.13	6.88 ± 0.10

* Constants were obtained by measuring transport of proline (0.1, 0.5 and 2.0 mM) after incubation of cells for 90 min with various concentrations of the hormonal agents, alone or in combination. Constants were estimated from best fit lines according to linear regression analysis and common intersections. Transport was measured as described in Materials and Methods. The estimated K_i was obtained by plotting $1/v$ vs $[I]$ according to Dixon [15] and the K_i' by plotting s/v vs $[I]$ according to Cornish-Bowden [16]. Data are presented as means ± S.E.M.

Table 3. Effects of estrogens and other agents on proline and leucine transport into R3230AC tumor cells*

Hormonal agent	Relative transport (% control)	
	Proline	Leucine
Estradiol-17β	59.3 ± 3.1	96.9 ± 3.9
Diethylstilbestrol	73.6 ± 1.5	97.1 ± 3.9
Tamoxifen	78.0 ± 2.6	103.5 ± 5.3
Estrone	83.3 ± 2.4	99.5 ± 5.9
Estriol	83.5 ± 2.6	98.1 ± 10.0
Estradiol-17α	89.0 ± 2.8	96.7 ± 1.7
Dexamethasone	66.9 ± 1.1	87.1 ± 5.9
Testosterone	83.5 ± 4.9	91.1 ± 5.3
Progesterone	81.5 ± 0.6	91.1 ± 3.6

* Dissociated cells from R3230AC tumors were incubated with various hormones (10^{-6} M) for 90 min. Transport of proline (0.1 mM) or leucine (0.1 mM) was measured as described in Materials and Methods. Data presented are means ± S.E.M. from three to six experiments. Transport into cells maintained under identical conditions but without addition of hormones represents controls and is equated to 100 percent; v_i for proline was 54.9 ± 4.1 pmoles · min^{-1} · 10^6 cells^{-1} (from forty-nine controls) and for leucine, the v_i was 47.3 ± 7.1 pmoles · min^{-1} · 10^6 cells^{-1} (from ten controls).

DISCUSSION

The data presented here confirm [11] and extend the observation that estrogens, and certain other steroids, significantly reduced entry of proline into R3230AC mammary tumor cells *in vitro*. For estradiol-17β, the most potent naturally occurring estrogen, reduction of proline transport exhibited both time- and dose-related characteristics. Kinetic analysis of the relationship between estradiol concentration and decreased proline transport suggested a relationship that would be expected of a non-competitive inhibitor. This was confirmed when the data were analyzed by Dixon and Cornish–Bowden plots; the common intersection on the x-axis would be expected for a non-competitive inhibitor [15, 16]. These data provided an estimated K_i of about 2 μM for estradiol-17β. All of the other estrogens studied, including the synthetic estrogen diethylstilbestrol, were less effective as inhibitors. Interestingly, the relative order for naturally occurring estrogens acting as inhibitors of proline transport resembled somewhat their order of potency obtained by bioassay. We reported a similar finding for the effects of estrogens on glucose transport in these tumor cells in primary culture [17]. It is not known whether these effects were mediated through estrogen receptors or were manifested directly, although the reported [18] presence of estrogen receptors in the

plasma membrane of uteri may be pertinent. However, this order of activity does not appear to be related to their relative partition coefficients, as would be assumed to occur in the lipophilic membrane [19].

Some subtle differences amongst these agents were detected from the Dixon plots. Estradiol-17β, diethylstilbestrol and tamoxifen yielded data compatible with a non-competitive inhibitor; a replot of these data (slope vs 1/[proline] gave a straight line not passing through the origin as expected for a non-competitive inhibitor. Dixon plots for estrone, estriol and estradiol-17α demonstrated a common intersection point slightly below the [*I*] axis; replots of these data, however, gave straight lines not passing through the origin. We concluded that all of these estrogens behaved as non-competitive inhibitors. Another relationship between biological activity and inhibitor potency was seen for the two isomers of estradiol. Both isomers, 17α-OH and 17β-OH, demonstrate similar solubility properties, with differences of about 1.5Å in the bond angles at the C-17 position. However, this is sufficient to drastically alter their biological activity and to demonstrate about a 4-fold difference in the estimated K_i.

Specificity of these effects was seen when comparing transport by the A system versus that by the L system. None of the estrogens significantly altered leucine transport at the same concentrations at which proline transport was decreased. Dexamethasone, however, did produce a small reduction in leucine transport, along with its ability to reduce proline entry, suggesting somewhat less specificity than that seen for estrogens. Glucocorticoids were reported to reduce growth of the R3230AC tumor [6]. It should be noted that tamoxifen, an anti-estrogen, demonstrated a capability to decrease proline transport. This anti-estrogen, like all others, possesses some agonist activities, depending on the target tissue examined. The results obtained for mixtures of estrogens and anti-estrogens suggest complex interactions between these agents, perhaps by allosteric effects on the carrier or other membrane components.

It is well known that phloretin and diethylstilbestrol can inhibit facilitated glucose transport [20], an action for diethylstilbestrol that was described by Burk *et al.* as "anti-insulin" [21]. The action of estrogens at pharmacological levels to inhibit amino acid transport by the A system offers another potential mechanism for their therapeutic action as agents in the treatment of advanced breast cancer; inhibition of transport would be compatible with reduction in tumor growth and/or induction of tumor regression. Weichselbaum *et al.* [22] examined cytotoxic effects of various steroids on a human breast tumor line, MCF-7. They reported no selectivity amongst the steroids and no cytotoxicity below 10^{-7} M. Interestingly, the estimated K_i for estradiol 17-β, $\sim 2 \times 10^{-6}$ M, reported here approximated that concentration causing a 50 percent cell kill in their assay. The effects of estrogens to inhibit growth of certain cells in culture [23] may reflect their ability to prevent uptake of substrates. Caution is needed, therefore, before attributing cytotoxic effects *in vitro* to receptor-mediated events.

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