Comparative Effects of Estrogen and Antiestrogens on Enzyme Activities in R3230AC Rat Mammary Tumors and Uteri of Tumor-bearing Animals

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ABSTRACT

Growth of the R3230AC mammary tumor, an ovarian-autonomous but estrogen-responsive tumor in Fischer 344 rats, is markedly depressed by administration of antiestrogen or high doses of estradiol. We have examined the effects of antiestrogens and estradiol on the activity of several enzymes known to be modulated by estrogen, namely, glucose-6-phosphate dehydrogenase (G6PD), NADP-dependent malic dehydrogenase (malic enzyme), α-glycerol phosphate dehydrogenase, and peroxidase. In addition, we have compared the effects of antiestrogens and estradiol on these enzymatic activities in mammary tumors and uteri of R3230AC tumor-bearing animals to enable a comparison of hormonal and antihormonal action in these two estrogen-sensitive tissues. Rats were treated with antiestrogen or estradiol for 24 days beginning on the day of tumor transplantation.

In mammary tumors, estradiol (25 µg s.c. in 0.15 M NaCl per day per rat) increased the activity of G6PD and malic enzyme 2-fold (expressed on a tissue weight or mg protein basis) and decreased the activity of α-glycerol phosphate dehydrogenase 2-fold; ovariectomy and antiestrogen treatments (250 µg s.c. in 0.15 M NaCl per day of 1-[2-p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxo]ethyl pyrrolidine hydrochloride (U11,100A) or of two related antiestrogens, cis-[3-p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol (U23,469) and α-(4-pyrrrolidinoethoxy)phenyl-4-methoxy-α'-nitrostilbene (CI-628) did not alter the activity from that of the diestrus control, and administration of antiestrogen along with estradiol blocked estradiol stimulation nearly completely. Tumor peroxidase activity was stimulated 5-fold by estradiol (25 µg) and 3-fold by antiestrogen (250 µg) in [2-p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl pyrrolidine hydrochloride above the levels in tumors of mature cycling animals. With concomitant administration of antiestrogen and estradiol, stimulation of peroxidase was reduced to the level seen with antiestrogen alone. In uteri of mammary tumor-bearing rats, some differences were seen. While estradiol significantly increased uterine weight and the activity of uterine G6PD (expressed on a tissue weight or mg protein basis), the activities of malic enzyme and peroxidase were not increased by estradiol above the levels seen in uteri of cycling animals. Ovariectomy and antiestrogen treatments, which markedly reduced uterine weight, suppressed the activities of these three enzymes far below the control level. When estradiol was administered along with antiestrogen, uterine weights and malic enzyme activity remained depressed. Estradiol stimulation of G6PD was reduced by antiestrogen, and uterine peroxidase activity returned to the high control level in antiestrogen plus estradiol-treated animals.

Hence, although antiestrogens and estrogens both reduce growth of R3230AC mammary tumors, the results indicate that their effects on tumor enzyme activities differ. Also, although the growth of both tumor and uterus is depressed by antiestrogen administration to mature cycling rats, the effects of antiestrogens on the activity of the same enzyme in tumor and uterus are frequently different.

INTRODUCTION

Synthetic estrogens and, more recently, antiestrogens have been used in the treatment of human breast cancer (4, 12, 13). The effectiveness of such endocrine therapy is positively correlated with the presence and content of estrogen receptors in mammary tumors; however, not all estrogen receptor-containing tumors are responsive to hormonal or antihormonal therapy (18, 23). Considerable current interest has focused on the development and exploitation of several mammary tumor systems in experimental animals that may serve as models for understanding the spectrum of hormone sensitivity of human breast cancers.

The R3230AC transplantable mammary tumor of the Fischer 344 rat, one such experimental animal model, was characterized initially by Hilf et al. (17). This mammary adenocarcinoma was found to grow equally well in ovariectomized or intact hosts, but continuous administration of high levels of estrogen or prolactin slowed the growth of these tumors (14, 16). Recently, our laboratory has shown that treatment with a variety of nonsteroidal antiestrogens also markedly depresses the growth of these ovarian-autonomous tumors (28).

Since growth of this mammary tumor is ovarian autonomous yet estrogen responsive, our aim in this study has been 2-fold: first, to examine and compare the effects of antiestrogens and estradiol on the activity of several enzymes known to be modulated by estrogen (2, 16), namely, G6PD,3 NADP-dependent malic dehydrogenase, called malic enzyme (L-malate:NADP oxidoreductase, decarboxylating); a-GOP, α-glycerol phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase); U23,469, cis-[3-p-(1,2,3,4-tetrahydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]-1,2-propanediol; U11,100A, 1-[2-p-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl pyrrolidine hydrochloride; CI-628, α-(4-pyrrrolidinoethoxy)phenyl-4-methoxy-α'-nitrostilbene; DMBA, 7,12-dimethylbenz(a)-anthracene.
estradiol on these enzymatic activities in mammary tumors and uteri of R3230AC tumor-bearing animals to enable a comparison of hormonal and antihormonal action in these 2 estrogen-sensitive tissues.

MATERIALS AND METHODS

R3230AC Rat Mammary Tumors. Forty-day-old female Fischer 344 rats (from Charles River Breeding Laboratories, Inc., North Wilmington, Mass.) received s.c. axillary transplants of R3230AC tumor via a sterile trocar technique under light sodium pentobarbital anesthesia. Twenty-one-day posttransplanted R3230AC tumors were removed from donor rats (kindly supplied by Mason Research Institute, Worcester, Mass.), minced, and kept in iced sterilized Medium 199 (containing penicillin and streptomycin) during tumor transplantation. Groups of rats either were ovariectomized 3 days before receiving tumor transplants or intact rats received vehicle (0.5 ml of 0.15 M NaCl containing 4% ethanol), various antiestrogens [U23,469 (100 µg), U11,100A (100 or 250 µg), and CI-628 (100 µg)], or estradiol plus antiestrogen (25 µg estradiol plus 100 or 250 µg U11,100A). The various compounds were given s.c. daily (in 0.5 ml 0.15 M NaCl containing 4% ethanol) for 25 days beginning on the day of tumor transplantation. Tumor palpation started 1 week after tumor transplantation and continued twice a week during the treatment period. Tumor size was measured in 2 dimensions (length x width) with calipers (28). At 25 days after tumor transplantation, the R3230AC tumor-bearing rats were sacrificed by decapitation, and their mammary tumors and uterus were collected, quickly frozen, and stored in a freezer at −85° until assayed. Control rats were either sacrificed at diestrus or without regard to their stage of the estrous cycle, as indicated in the chart legends.

Chemicals and Reagents. All enzyme substrates, NADP, NADH, and 17β-estradiol were obtained from Sigma Chemical Co., St. Louis, Mo. The antiestrogens U23,469 (Isomer A) and U11,100A (nafinoxide HCl) were kindly provided by the Upjohn Co., Kalamazoo, Mich., and CI-628 was kindly provided by the Parkes, Davis & Co., Ann Arbor, Mich.

Enzyme Activity Assays. R3230AC tumors and uteri were homogenized (100 mg/ml) in 0.01 M Tris-HCl buffer, pH 7.4, and assays for 3 of the enzymes were performed on the homogenate supernatant (20,000 x g for 30 min). Assays were carried out under conditions showing zero-order kinetics, and the absorbance change at 340 nm due to the production of NADPH or the oxidation of NADH was monitored. The enzymes monitored were G6PD, by the method of Glock and McLean (11), α-GPD, by the procedure of Beishenherz et al. (3) modified by using dihydroxyacetone phosphate as the substrate and measuring the oxidation of NADH as described by Cohen and Hilt (6), and NADP-malate dehydrogenase, decarboxylating (malic enzyme) by the method of Ochoa et al. (25).

Tumor peroxidase activity was solubilized, as described by Lyttle and DeSombre (22), by rehomogenizing the sediment (40,000 x g for 30 min) with 10 mM Tris-HCl, pH 7.2, containing 1.0 M CaCl2. Uterine peroxidase activity was solubilized with 10 mM Tris-HCl, pH 7.2, containing 0.5 M CaCl2. Solubilization of peroxidase activity of uterus and tumor was monitored with different concentrations of CaCl2 and was found to be complete with the CaCl2 concentrations stated above. No peroxidase activity was found in the 40,000 x g supernatant fraction. Peroxidase activity was determined exactly as described by Lyttle and DeSombre (22). The assay mixture (3.0 ml total volume) contained 13 mM guaiacol, 0.3 mM H2O2, and an aliquot of sample extract. The rate of oxidation of guaiacol was measured in the presence of H2O2 as indicated by a change in absorbance at 470 nm.

Statistics. The significance of differences between treatment groups and the control group was examined by Student’s t test.

RESULTS

Effects of Estrogen and Antiestrogen on Enzyme Activities in R3230AC Mammary Tumors. Since in previous publications (19, 28) we found that antiestrogens and estrogen diminished the rate of R3230AC mammary tumor growth, we decided to determine if these compounds affected R3230AC tumor enzyme activities in a parallel manner. For these studies, either intact Fischer 344 rats were treated with antiestrogen (250 µg), estradiol (25 µg), or vehicle control daily for 24 days beginning on the day of tumor transplantation or tumors were transplanted into females that had been ovariectomized 3 days prior to the time of tumor transplantation. When harvested at 25 days after transplantation, tumors were much smaller in antiestrogen-treated and estradiol-treated host animals, while tumor size was similar in control and ovariectomized hosts. These tumor growth profiles have been published previously (28). The effects of these hormonal-antihormonal treatments on the activities of several metabolic enzymes in mammary tumors are shown in Chart 1. Estradiol increased the activity of G6PD and malic enzyme 2-fold (expressed on a tissue weight or mg protein basis) and decreased the activity of α-GPD 2-fold, in keeping with previous reports (14—17). Antiestrogen treatment (CI-628, U23,469, or U11,100A) and ovariectomy did not change the activity of these enzymes from that of the diestrus control. The concomitant administration of antiestrogen along with estradiol greatly diminished the estradiol-stimulated increase in G6PD activity and completely blocked the increase in malic enzyme activity seen with estradiol alone (Chart 1). Hilt

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(15) has reported a similar depression of estradiol-stimulated increases in these enzymes by the antiestrogen MER-25.

Peroxidase is an enzyme whose activity has been shown to be markedly increased by estrogen in hormone-dependent DMBA-induced rat mammary tumors and in uteri of several animal species (8, 21). Hence, we examined the effects of hormonal manipulation on peroxidase activity in this ovarian-autonomous mammary tumor. Our findings are reported in Chart 2.

Peroxidase activity was found to be low in mammary tumors of control rats and was increased 5-fold following treatment with a high dose (25 μg) of estradiol. Antiestrogen treatment increased peroxidase activity 3-fold, and when antiestrogen was administered along with estradiol, mammary tumor peroxidase activity was only stimulated 3-fold, namely, to the level seen with antiestrogen alone. Ovariectomy had no effect on the level of peroxidase activity in tumors (Chart 2).

Effects of Estrogen and Antiestrogen on Enzyme Activities in Uteri. In uteri of mammary tumor-bearing animals, daily administration of estradiol (25 μg/day) increased uterine weight above that of cycling females, while ovariectomy or antiestrogen administration resulted in a marked decrease in uterine size (Chart 3). When antiestrogen was administered with estradiol, uterine weights were greatly depressed, but not to the extent seen with antiestrogen alone (Chart 3). Uterine weights were consistently lower in antiestrogen-treated animals than in ovariectomized Fischer rats, although body weights did not differ significantly between treatment groups. This differs from the situation in Sprague-Dawley rats bearing DMBA-induced mammary tumors in which similar daily antiestrogen administration decreased uterine weights below those of controls, but never to the extent seen in ovariectomized animals (27). This may suggest a significant adrenal gland contribution in these Fischer 344 rats, a possibility that we have yet to explore.

Chart 4 reports the effects of these endocrine manipulations on the activities of various enzymes in uteri. Estradiol increased the activity of uterine G6PD, while ovariectomy or antiestrogen administration significantly depressed this activity. These depressive effects of ovariectomy and antiestrogen on G6PD activity in uteri are in contrast to the lack of effect of these treatments on G6PD activity in R3230AC mammary tumors. As seen in Chart 4, when antiestrogen treatment (100 or 250 μg U11,100A per day) was combined with estrogen treatment (25 μg/day), uterine G6PD activity was found to be equivalent to the level seen in control uteri.

The activity of another uterine enzyme, malic enzyme, appeared to be increased following estrogen treatment, but not to a level that was statistically different (p < 0.05) from controls (Chart 4). Nevertheless, ovariectomy and antiestrogen treatment significantly depressed malic enzyme activity in uteri. Malic enzyme activity also remained depressed when antiestrogen treatment was combined with estrogen treatment.

Peroxidase activity (Chart 5) was found to be high in uteri of control cycling animals, and it was not stimulated further by the administration of estradiol. Ovariectomy or antiestrogen treatment almost completely abolished peroxidase activity, but, when antiestrogen was administered together with estradiol, uterine peroxidase activity returned to the high level seen in control and estradiol-treated females (Chart 5).

DISCUSSION

These results indicate significant differences in the responses of enzymes of the R3230AC mammary tumor to estrogen and antiestrogen, even though both endocrine treatments give similar depression of the growth rate of these mammary tumors. Also, although the growth of uterus as well as tumor is depressed by antiestrogen, the effects of antiestrogen on the activity of the same enzyme in tumor and uterus are frequently
There is considerable evidence that the enzyme peroxidase is a meaningful marker of estrogen-induced responses in reproductive tissues. Peroxidase has been identified histochemically in the normal rat mammary gland (1, 26) and in the DMBA-induced mammary tumor (7), and it has been shown to be localized in epithelial cells. We believed, therefore, that peroxidase might serve as an appropriate marker of estrogen responsiveness in the R3230AC tumor, which is epithelial in nature. Our results demonstrate a low level of peroxidase activity in control tumors that is stimulated 5-fold by estrogen treatment. U11,100A was a weak agonist in this assay, since it stimulated peroxidase activity over 2-fold. Interestingly, when U11,100A was administered together with estradiol, it antagonized the ability of estradiol to stimulate peroxidase activity in mammary tumors, in contrast to its lack of antagonism of estradiol action at similar doses in the uterus. In some tissues such as rat uterus, some effects of antiestrogen versus estradiol have been attributed to differential stimulation of different cell types by the 2 agents (5). In this R3230AC tumor, which consists almost exclusively of epithelial cells, the effects of estradiol and antiestrogen could not be explained by differential cell stimulation.

Peroxidase activity was found to be high in uteri of control cycling rats, and, while not being significantly increased by the administration of a high (25 µg) estradiol dose, uterine peroxidase activity was drastically reduced by ovariectomy or antiestrogen. These findings are in agreement with those of D’Sombre and Lyttle (9), who have shown that, while total uterine peroxidase activity increases during estrus, peroxidase activity does not change in uteri during the cycle when expressed on a tissue weight basis. In the present study, the low level of peroxidase activity in uteri of ovariectomized or antiestrogen-treated females suggests that this enzyme is very sensitive to endogenous hormone, such that the endogenous physiological levels of estrogen in cycling rats maximally stimulate its activity. However, while antiestrogen (250 µg, Chart 5; or 100 µg, not shown) was able to effectively antagonize the endogenous estrogen in control cycling rats, as monitored by inhibition of peroxidase, similar antiestrogen doses were not able to antagonize pharmacological (25 µg/day) doses of estradiol. Presumably, the ratio of antiestrogen to estrogen would have to be much higher than the one we used to prevent estrogen from stimulating uterine peroxidase activity. However, this ratio of antiestrogen to estrogen was effective in reducing uterine weight and uterine malic enzyme activity (Charts 3 and 4).

Of note are the marked quantitative differences in the basal activities and stimulated levels of peroxidase in mammary tumor and uterus. Although the activities of G6PD and malic enzyme were similar (on a protein, DNA, or tissue weight basis) in uterus and R3230AC tumor, the activity of peroxidase in R3230AC tumors was only 0.5% that seen in uteri from the same control animals. A comparison between the levels of enzyme activities in the R3230AC tumor (this paper) and those reported for the DMBA-induced mammary tumor (8) reveals that there is 10 times as much peroxidase activity in the DMBA tumor as in the R3230AC tumor. High levels of peroxidase activity are also found in human breast cancer samples, and a qualitative correlation between the presence of estrogen receptor and peroxidase activity has been shown in human breast tumors (10, 21).

The results we have found here are consistent with the
suggestion that the responsiveness of a target organ to estrogen can be correlated with the presence of the estrogen receptor. The R3230AC tumor contains estrogen receptor with a high affinity for estradiol \( K_d = 2 \times 10^{-10} \text{ M} \) (24, 29), similar to that found in DMBA tumors (24) and normal lactating mammary gland (29); but receptor levels are only 10 to 30% the level found in DMBA tumors (24, 27, 28) or lactating mammary gland (29). In addition, estrogens as well as antiestrogens interact with the cytoplasmic receptor in R3230AC tumors and move the receptor into the nuclear compartment (28). It has been proposed that the estrogen sensitivity, but lack of ovarian dependence, of the R3230AC tumor may be associated with this low estrogen receptor level (24). Nonetheless, the observation that estrogen and antiestrogens interact with estrogen receptors in R3230AC tumors and modulate enzyme activities and tumor growth rate indicate that this tumor is clearly estrogen responsive, although not ovarian dependent, for its growth. This tumor has also been shown to respond to estrogen with lactation-like morphological and biochemical changes and to be prolactin sensitive. However, the possibility that the differing enzyme results in tumor and uterus might be due to prolactin seems unlikely. At the doses used in this study, the various antiestrogens did not alter serum prolactin levels (28). In addition, Hilf et al. (17) have reported that estrogen stimulation of tumor G6PD and malic enzyme occurred in the presence or absence of high levels of prolactin.

The present results indicate clear differences in the sensitivities of several enzymes to estrogen and antiestrogen and emphasize that the effects of antiestrogens on the activity of the same enzyme in tumor and uterus are frequently different, although the growth of both tumor and uterus is similarly depressed by antiestrogen treatment. These considerations would seem important in interpreting the varied effects of antiestrogens, when given therapeutically, in different estrogen target tissues.

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REFERENCES

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