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ESTROGEN INDUCED SYNTHESIS OF SPECIFIC PROTEINS IN HUMAN BREAST CANCER CELLS

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SUMMARY: Human breast cancer cells in tissue culture (MCF-7) were pretreated with the antiestrogen nafoxidine to arrest cellular proliferation and then were given estradiol to release this block and stimulate DNA synthesis and cell division. During this period of growth stimulation intracellular proteins, labeled by a double isotope method, were analyzed on SDS-polyacrylamide gel electrophoresis. Estradiol directly increases the rates of synthesis of specific proteins which migrate on SDS-gels at molecular weights of 24,000 and 36,000. Nafoxidine-pretreatment alone does not induce these same proteins, and no changes in the rates of specific protein synthesis occur in cells grown on control medium for the same length of time as on estradiol. Induced synthesis of these proteins is observed only during the period of estrogen stimulation of cell proliferation following pretreatment with nafoxidine. We do not detect induction when cells are incubated with estradiol without antiestrogen-pretreatment. Since rescue of antiestrogen growth inhibition is also the only condition under which MCF-7 cell division can be reproducibly stimulated by estrogen, these proteins may be related to estrogen effects on cellular proliferation.

The presence of estrogen receptors (ER) in human breast cancer is undoubtedly of clinical value in predicting the sensitivity of tumors to estrogens. However, only about 60% of those tumors containing ER will respond to endocrine manipulations designed to reduce endogenous estrogens (1). Since progesterone receptor (PgR) is unequivocally regulated by estrogens in human breast cancer cells in tissue culture, PgR has been theorized to be a marker of tumor sensitivity to estrogens (2, 3). The simultaneous measurement of ER and PgR in fact clearly improves the reliability of ER measurements in predicting tumor responsiveness to endocrine therapies (4). The correlation, however, between the presence of steroid receptors and tumor response to endocrine manipulations is still far from perfect. Therefore, other

ABBREVIATIONS USED: MEM: minimum essential medium; HEPES: N-2-Hydroxyethlpiperazine-N′-2-Ethanesulfonic Acid; SDS: Sodium dodecyl sulfate; Estradiol: 1, 3, 5 (10)-estratriene-3, 17β-diol; ER: Estrogen receptor; PgR: Progesterone receptor.

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biochemical markers of estrogen action are needed to fill the gap in our understanding of how estrogens influence breast tumors.

The MCF-7 human breast cancer cell line is a good in vitro model for studying estrogen action in breast cancer since the cells contain a functional ER (5) and also contain PgR (6) which is regulated by estrogen (2). We have examined estrogen effects on protein synthesis in MCF-7 cells by analyzing intracellular proteins on SDS- polyacrylamide gel electrophoresis using a double isotope method of labeling soluble proteins (7). We find that when cell growth is arrested by the antiestrogen nafoxidine and subsequently released by estradiol, synthesis of certain specific proteins is induced during the period of maximal stimulation of cell proliferation.

MATERIALS AND METHODS

Chemicals: Leucine, L-[3,4,5-^{3}H(N)] (>100Ci/mmol) and Leucine, L-[^{14}C(U)] (>300mCi/mmol) were obtained from New England Nuclear. Estradiol-17β was purchased from Calbiochem and nafoxidine (1-(2-p-(3,4-dihydro-6-methoxy-2-phenyl-1-napthyl) phenyoxyethyl) pyrrolidine hydrochloride; U11-100A) was a gift from Upjohn.

Cell Culture: MCF-7 cells, a gift of Dr. Herbert Soule (Michigan Cancer Foundation) were plated in Corning plastic flasks (75 sq. cm.) at a density of 1.25 x 10^{6} cells/flask and grown in 5% CO_2 in air at 37°C. Growth medium consisted of Eagle's minimum essential medium (MEM), 1% nonessential amino acids (Gibco), 2 mM L-glutamine (Gibco), 6 ng/ml insulin (Sigma), 10% calf serum (Gibco), 25 ng/ml gentamicin (Schering), 10 mM HEPES buffer and 0.05% $NaHCO_3$. Two days after plating, growth medium was replaced with experimental medium consisting of MEM, 1% nonessential amino acids, 2 mM L-glutamine, 25 ng/ml gentamicin, and 10 mM HEPES. Experimental medium also contained 5% calf serum stripped of endogenous steroids by treatment with dextran coated charcoal (2). Steroid hormones (estradiol and nafoxidine) were prepared in 1000 fold concentrated solutions in ethanol and added to experimental medium (0.5 ml/500 ml of medium) to obtain the final concentrations described in experiments. Control medium contained 0.5 ml ethanol/500 ml. The medium was changed every 48 hrs. Cells were harvested by a 10 min incubation at 37°C with 1 mM EDTA in Ca^{++}, Mg^{++}-free Hanks balanced salt solution.

Cells were then pelleted by a low speed centrifugation and washed once with Hanks solution at 4°C and once with 10 mM sodium phosphate at pH 7.4.

Labeling of Proteins: Cells treated with estradiol were pulsed for 2 hr at 37°C with 10 uCi/ml of [^{3}H]-leucine in the same medium used for hormone incubations with the exception of using leucine-free MEM (Gibco). Cells that served as controls were pulsed for 2 hr at 37°C with 2.5 uCi/ml of [^{14}C]-leucine.

Analysis of Induced Proteins: After pulsing with radiolabeled leucine, cells from 2 flasks of each estrogen treated and control group were mixed together in a hypotonic medium (1.5 mM $MgCl_2$), allowed to swell and then homogenized with a Teflon-glass Potter-Elvehjem homogenizer. Immediately following homogenization, an equal volume of 10 mM sodium phosphate pH 7.4 was added and mixed with the homogenate. Homogenates were then centrifuged at 105,000 xg for 30 min. Aliquots of the supernatant were diluted fourfold with an SDS-buffer consisting of 1% SDS, 0.0625 M Tris-HCl, pH 6.8, 6 M urea, 5 mM β-mercaptoethanol and 0.01% bromphenol blue, and heated for 5 min at 90°C. Approximately 50ug of protein from each denatured aliquot was submitted to polyacrylamide gel electrophoresis in SDS.

SDS-electrophoresis was on a discontinous slab gel system containing 10% polyacrylamide in the separating gel and a 3% stacking gel as described by Laemmli (8) with the exception that SDS was present only in the upper reservoir buffer at 0.03% and that the electrode buffer contained 0.025 M Tris base and 0.192 M glycine. Proteins in gel slabs were fixed for 1 hr in 40% methanol, 10% acetic acid and stained overnight with Coomassie Brilliant Blue R-250. Each gel lane was sliced into approximately 85 1-mm pieces which were swelled for 24 hrs at 37° in 5ml of scintillation fluid containing 4gm of PPO, 0.05g POPOP/liter toluene and 3% Protosol (New England Nuclear) before counting. $^{3}H/^{14}C$ ratios in each gel slice were calculated after converting cpm to dpm and correcting for the contribution of ^{14}C counts in the ^{3}H-channel. Estrogen-induced proteins were detected in the gels by a peak over the baseline in the $^{3}H/^{14}C$ ratio. Basal values for the $^{3}H/^{14}C$ ratio were determined as the average of the ratio in every slice across the gel. The molecular weights of induced proteins were estimated by their electrophoretic mobilities relative to protein standards.

Miscellaneous: Protein concentrations in cytosols were determined by the method of Lowry et al. (9) and total DNA/flask by the diphenylamine method of Burton (10).

RESULTS

Proliferation of our MCF-7 cells is not reproducibly enhanced by addition of estradiol to the growth medium. However, incubation with antiestrogens such as tamoxifen or nafoxidine inhibits cellular proliferation and this inhibition can be rapidly reversed by estradiol (5, 11, 12). We have analyzed protein synthesis in MCF-7 cells during this period of estrogen reversal of growth inhibition. Growth conditions under which we examined protein synthesis are shown in Figure 1. Cells were grown for 6 days in the continuous presence of 1uM nafoxidine and then switched

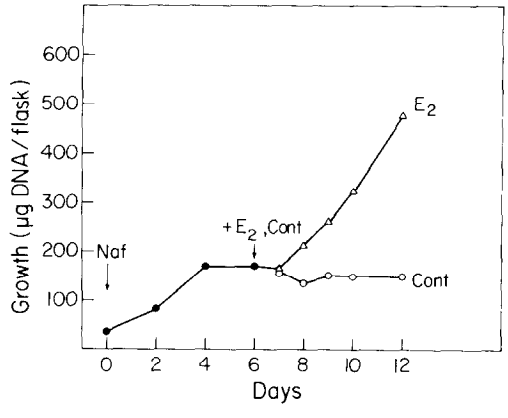

Figure 1. Antiestrogen effect on cell growth and reversal with estradiol. Two days after plating, cells were maintained on 1.0 uM (Naf) nafoxidine (●) in experimental medium for 6 days, then changed to medium containing either 0.01% ethanol vehicle (control), (o) or 10 nM (E_2) estradiol (Δ) and grown for an additional 6 days. Total DNA per flask was determined at the times indicated. Values represent the mean determinations from duplicate T-75 flasks.

to either control medium or medium containing 10 nM estradiol. By day 6, nafoxidine has arrested cellular proliferation, and changing at this time to control medium is without effect on growth. Growth inhibition, however, is rapidly overcome by incubating with estradiol. Radiolabeled proteins were analyzed by SDS-gel electrophoresis after 4 days of treatment of nafoxidine-inhibited cells with estradiol. Proteins extracted from estrogen treated cells were labeled with (^{3}H)-leucine while proteins from nafoxidine-pretreated cells served as controls and were labeled with (^{14}C)-leucine. The results of a representative experiment showing the ^{3}H and ^{14}C dpms in each gel slice is illustrated in Figure 2. A pronounced increase in the ^{3}H/^{14}C ratio over

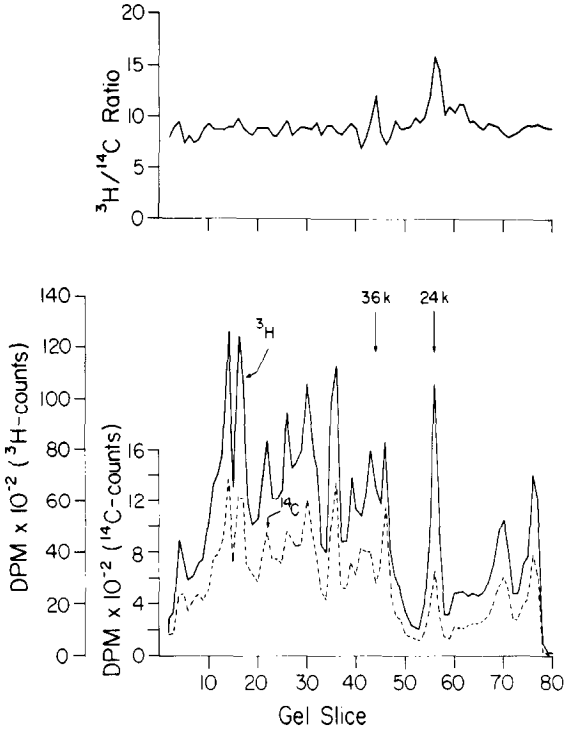

Figure 2. SDS-gel electrophoresis of proteins synthesized by nafoxidine-treated cells (^{14}C) and cells rescued from nafoxidine inhibition by estradiol (^{3}H). Two days after plating cells were incubated continuously for 6 days with growth medium containing 1.0 uM nafoxidine. At the end of 6 days pretreatment with nafoxidine, 2 T-75 flasks were continued on nafoxidine for another 4 days and two flasks were changed to medium with 10 nM estradiol for 4 days. Nafoxidine-treated cells served as controls and were labeled for 2 hr with (^{14}C)-leucine, while estrogen-treated cells were labeled for 2 hrs with (^{3}H)-leucine. Equal numbers of control and estrogen-treated cells were mixed together, homogenized and run on SDS-gels. The arrows indicate the positions and molecular weight estimates of the induced ^{3}H/^{14}C ratio peaks. The upper panel shows the ratio ^{3}H/^{14}C dpms in each gel slice. The bottom panel shows the total dpm in each gel slice for both ^{3}H and ^{14}C.

baseline was detected at a molecular weight of about 24,000 daltons. A distinct but smaller increase was detected at a molecular weight of 36,000 daltons. Data from several independent experiments showed a mean value of 24,183 (range, 23,202-26,467) and 35,510 (range, 34,455-37,638) daltons respectively for these estrogen-induced ratio peaks. No substantial or consistent increase over the basal values were observed in the rest of the gel.

The relative prominence of the two induced peaks seemed to vary with each experiment. By way of example, the $^{3}H/^{14}C$ ratios measured in a separate but identical experiment at 4 days of estrogen treatment are shown in Figure 3 (upper panel). Here the major induced peak is at 36,000 daltons which shows about a 2 fold increase over baseline compared with the induced peak at 24,000 daltons which shows an increase of about 1.5 fold. We have estimated these relative increases in seven independent experiments, shown in Table I. The results confirm that considerable variation occurs not only in the magnitude of the induction, but also in which protein

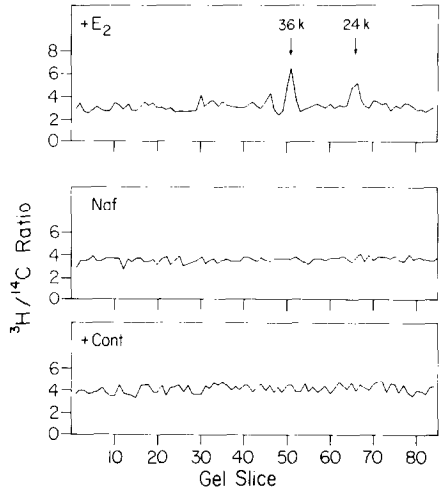

Figure 3. SDS-gel electrophoresis of labeled MCF-7 cytosol proteins. Upper panel ("+ E_2"): the experimental protocol was the same as described in Figure 2. This is an identical but independent experiment. Middle panel ("Naf") cells were grown for 6 days on 1.0 uM nafoxidine and 2 flasks were labeled with (^{3}H)-leucine and two with (^{14}C)-leucine. Proteins from both groups of nafoxidine-treated cells were run simultaneously on one gel. Lower panel ("+ cont"): at the end of 6 days incubation with 1.0 uM nafoxidine, cells were either continued for 4 days on nafoxidine and then labeled with (^{14}C)-leucine or changed to control media for 4 days and then labeled with (^{3}H)-leucine. Proteins from both labeling groups were run simultaneously on the same gel.

Table 1

Magnitude of Estrogen Induction of Specific Protein(s)

Experiment (n=7)[1]	Fold Increase in $^{3}H/^{14}C$ Ratio[2]	
	24,000 m.w.	36,000 m.w.
1	1.50	1.64
2	1.24	1.65
3	1.44	1.88
4	1.81	1.34
5	1.53	1.31
6	1.71	1.69
7	1.57	1.71
mean (n=7)	1.54	1.60

1. The values given were calculated from seven, independent but identical experiments. Experimental conditions were the same as described in Figure 2.
2. The fold increase was calculated by determining the increase in the $^{3}H/^{14}C$ ratio peak at 24,000 and 36,000 daltons over the baseline. Baseline was determined to be the mean value of the ratio for all gel slices excluding the induced bands.

band is induced to the greater extent. This is apparently due to variable effects either in the cell's response to estrogen or in extraction of these proteins and not due to inconsistencies in gel electrophoresis, since frozen aliquots of the same labeled cytosol proteins re-run on SDS-gel give very consistent $^{3}H/^{14}C$ ratio patterns. The mean increase from separate experiments (N=7) was 1.54 fold at 24,000 molecular weight and 1.6 fold at 36,000 molecular weight.

In control experiments, proteins were extracted and coelectrophoresed on SDS-gels from cells incubated only with nafoxidine and labeled with both isotopes. Proteins were also extracted and coelectrophoresed from nafoxidine-treated cells (pulsed with [^{14}C]-leucine) and from nafoxidine-pretreated cells changed to control medium for 4 days (pulsed with [^{3}H]-leucine). These control experiments (Figure 3, middle and lower panel) show no ratio peaks indicating that the increased ratio observed at 24,000 and 36,000 molecular weight in the previous experiment is the result of estradiol stimulation.

DISCUSSION

We have observed that estradiol in MCF-7 human breast cancer cells directly stimulates synthesis of specific proteins which migrate on SDS-gel electrophoresis at molecular weights of 24,000 and 36,000 daltons. Induction of these proteins does not occur following nafoxidine treatment or in cells maintained on control growth medium for the same length of time as on estradiol. We have consistently detected induction of these proteins by the double isotope uptake method in several independent experiments.

Induction of specific protein synthesis is observed only during the period of estrogen stimulation of cells whose growth has been arrested by the antiestrogen nafoxidine. We do not detect induction of these proteins when cells grown on control medium are treated with estradiol alone (unpublished data). Growth of MCF-7 cells in our hands also does not appear to be reproducibly influenced by estrogen alone (11), and we believe that cells maintained on control growth medium (medium freed of endogenous steroids) may be proliferating at maximal rates in the absence of estrogens. These estrogen-induced proteins therefore may be related to estrogen effects on cell growth, since estrogen rescue after antiestrogen growth inhibition is the only condition under which we observe either the induction of specific protein synthesis or growth stimulation. To further test this hypothesis we are currently investigating whether estrogens induce these same proteins in other human breast cancer cell lines, some of which show different levels of sensitivity in their growth to antiestrogens and estrogens.

We do not know the nature or identity of these estrogen-induced proteins, although their molecular weights are different from that of the rat uterine induced protein (13), and the time course of their induction is much slower (7). Lactate dehydrogenase (15), however, from various mammalian tissues can be dissociated into subunits of molecular weight 36,000 and the milk protein casein (16) has a molecular weight of about 24,000. MCF-7 cells contain only the 5th isozyme of lactate dehydrogenase (LDH-5), whose activity is stimulated 2-fold by estradiol (17). Synthesis of casein by MCF-7 cells has not been demonstrated.

Westley and Rochefort (14) recently demonstrated that estrogens induce an MCF-7 cell secretory protein, found only in the medium, which constitutes a major fraction of all secretory proteins. By two dimensional electrophoresis they were also able to show that estrogen regulates several intracellular proteins. The molecular weights of these estrogen-induced proteins described by Westley and Rochefort (14), however, are higher than those of the proteins detected in our studies.

We have estimated the magnitude of protein induction (Table 1), by monitoring changes in the rates of uptake of radiolabeled leucine. This method, however, only gives a minimum estimate because extraneous proteins may contribute to the total counts contained in each protein band in the gel. Coomassie stained SDS-gels of cytosol proteins, extracted from cells pretreated with antiestrogen followed by 4 days of estradiol show a prominent band at the Rf for 24,000 molecular weight proteins. We have determined by directly cutting out and counting this band that it contains the estrogen-induced ratio peak. The intensity of this stained band and the relative number of counts incorporated into protein contained in this band (Figure 2) indicate that the 24,000 molecular weight protein whose synthesis is stimulated by estradiol may represent a significant amount (>1%) of total intracellular proteins. In order to determine to what extent extraneous proteins contribute to the staining of the 24,000 molecular weight band and in turn to accurately estimate the percentage of total intracellular protein contained in the estrogen regulated protein(s) will require isolation of a homogenous band containing the induced ratio peak. We cannot detect a stained protein band on SDS-gels directly corresponding with the 36,000 molecular weight ratio peak, presumably because it comigrates to a position on the gel with several other proteins. This estrogen-induced ratio peak, in fact, does not coincide with a major peak of total radioactivity but is located on the shoulder of a peak (Figure 2).

These estrogen regulated proteins in MCF-7 cells may be excellent markers of estrogen action in human breast cancer. If these specific proteins are present in the abundance indicated by our preliminary findings, they may be the specific probes needed to permit isolation of hormone regulated messages and to further study estrogen regulation of gene expression in breast tumors.

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