Estrogen Control of Progesterone Receptor in Human Breast Cancer

CORRELATION WITH NUCLEAR PROCESSING OF ESTROGEN RECEPTOR

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Some human breast cancers which contain estrogen receptors (ER) also contain progesterone receptors (PgR), whose presence may indicate that the ER remain responsive to estrogen and can still control specific protein synthesis. To demonstrate a direct role for ER we have used the human breast cancer cell line, MCF-7, which contains ER and low PgR levels. We have studied the effects of addition and withdrawal of estradiol on PgR synthesis and correlated this with ER binding, translocation, nuclear processing reactions, and restoration of unoccupied ER.

In cells treated 4 to 5 days with estradiol (0.001 to 100 nM), basal PgR levels (0.3 to 0.7 pmol/mg of DNA) increase 3- to 6-fold. The PgR response is dose-dependent; 0.1 nM estradiol, a physiological dose, is maximally effective. Growth and induction of PgR by 0.1 nM estradiol are suppressed by an antioestrogen Tamoxifen at 10,000-fold molar excess (1 μM), but reversed by supraphysiological estradiol (10 nM) which reduces the molar excess of Tamoxifen to only 100-fold.

In MCF-7, ER unoccupied by hormone is not restricted to the cytoplasm (Rc) since a portion of the cellular receptor can also be found in the nucleus in unoccupied form (Rn). The level of PgR induction correlates with the extent of Rc binding and translocation and also with the extent of Rn binding. At 0.1 nM estradiol, 85% of Rc and Rn are depleted and PgR is maximally stimulated. The levels of PgR also parallel processing of bound estrogen receptor (RnE) appearing in the nucleus. Processing, during which a new steady state level of RnE is achieved, appears to be a saturable event. At low estradiol doses, despite Rc and Rn binding of estradiol, RnE fails to accumulate and total ER decreases. The loss is greatest at 0.1 nM estradiol.

Although at higher doses the nuclear receptor remains unprocessed and RnE levels increase. The processing effect is rapid, beginning within 10 min of estradiol addition and completed by 5 h. Processed RnE levels are seen prior to PgR induction and during maintenance of induced PgR states. In estrogen withdrawn cells, PgR fall in parallel with end of processing and restoration of Rc and Rn.

Since the original identification of cytoplasmic estrogen receptors (ER) in human breast cancer (1, 2), rapid progress has been made toward linking the presence of the receptor with endocrine responsiveness of the tumor. It is now known that the likelihood of a successful response to endocrine therapy is increased at least 10-fold in patients whose tumors are positive for ER (3). However, not all ER containing tumors respond, and this has led to the concept that ER are necessary but not sufficient markers of hormone dependence.

We have demonstrated progesterone receptors (PgR) in human breast tumors (4) and have proposed that this receptor, whose synthesis is known to be controlled by estrogen in the uterus, might serve as a marker of estrogen action in breast cancer (5). Thus, the presence of PgR in a tumor would indicate that the entire sequence involving estrogen binding to cytoplasmic receptor, movement of the receptor complex into the nucleus, and stimulation of a specific end product can be achieved in the tumor cell and would rule out the existence of a defect beyond the binding step.

Although this proposal assumes that PgR are under control of estrogen acting through ER, this priming effect has never been demonstrated in human breast cancer. That PgR synthesis is mediated by the estrogen receptor system has not been described for any tissue.

The MCF-7 cell line derived from a patient with metastatic breast cancer (6) is ideally suited to study the mechanism of PgR induction. These cells are in permanent tissue culture, contain estrogen receptors (7, 8), and are estrogen-responsive (9). Cells grown without estradiol have only basal PgR levels (8). We have shown that MCF-7 cells have an unusual estrogen receptor distribution: unfilled receptor sites are present in the cytoplasm (Rc) and nucleus (Rn) (10,11). Such a receptor distribution has also been found for other cells in culture (12) and in solid human breast tumors as well (13). It is unknown whether Rn possesses biological activity. We have

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‡ These abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; Rc, unoccupied cytoplasmic estrogen receptor; Rn, unoccupied nuclear estrogen receptor; RnE, occupied nuclear estrogen receptor; MEM medium, Earles-based minimal essential medium; DCC, dextran-coated charcoal.
speculated that Rn could be inducing cell growth and division in the absence of estrogen, in view of a demonstrated (9, 10) independence of these cells on estrogen for growth.

In this report, we show that in these human breast tumor cells, PgR are under estrogen control and that PgR synthesis involves the estrogen receptor system. We have studied the effects of addition and removal of continuous estradiol on PgR synthesis and correlated this synthesis with Rc and Rn binding, translocation, nuclear processing reactions, and restoration of unoccupied ER.

EXPERIMENTAL PROCEDURES

Chemicals – Radioactive [2,4,6,7-3H]estradiol-17β (100 Ci/mmol) was obtained from New England Nuclear. R5020 [6,7-3H]R5020, [1,2,3,-3H117,21-flasks (75 cm²) or at 5 x 10^6 cells in glass roller bottles (692 cm²) and serum). This procedure removed more than 96% of a trace amount of mine, 0.006 ng/ml of insulin, 10 nM hydrocortisone, 1 pg/ml of 4', 10 nM thioglycerol, and 10% glycerol).

5% calf serum stripped of endogenous hormones by 30-min incubations described in the text. Flasks were fluid changed with fresh to 30-min incubation at 37°C with 1 mM EDTA in Ca²⁺/Mg²⁺-free minimal essential medium prepared in Earle's salts (MEM, Gibco) minimal medium consisted of Eagle's

mitochondria was less than 2% of the total DNA.

The nuclear pellet was resuspended in 1 to 3 ml of Tris buffer (10 mM, pH 7.4) containing 0.6 M KCl and incubated for 1 h during which time the pellet was resuspended every 15 min.

Solubilized proteins were then obtained by centrifugation at 105,000 x g for 30 min. DNA was used for calculation of cytoplasmic and nuclear receptors.

Single Saturating Dose ASSAY (Estrogen Receptor) – Unoccupied cytoplasmic and nuclear receptors (Rc and Rn) were determined by incubation of estradiol precipitates at 0°C for 20 h with 5 nM [3H]estradiol. Total (estradiol)-filled plus un-filled sites were determined by incubation of cytosol or nuclear extracts at 30°C for 5 h with 10 nM [3H]estradiol. Nonspecific binding was determined by parallel incubations with [3H]estradiol plus 100-fold excess diethylstilbestrol. The difference between total (30°C) and un-filled sites (4°C) was used to determine the value for estradiol-filled sites (Rc and Rn). Preliminary studies showed the levels of Rc were negligible even after 24 h of exposure to estradiol, thus this component was not routinely assayed. The concentrations of [3H]estradiol used saturate 95 to 100% of receptor with minimal (5 to 10%) nonspecific binding (11). Following incubation, tubes were washed twice with phosphate buffer, extracted, and counted directly in 5 ml of toluene-based scintillation fluid (14.0 g of 2,5-diphenyloxazole (POPOP), 0.05 g of 1,4-bis(2-methylphenoxy)benzene (POPPO), and 1 liter of toluene) in a Beckman LS 233 counter with a counting efficiency of 50%

Protamine Sulfate Assay (Estrogen Receptor) – Concentrated cytosol (200 μl) were precipitated with 250 μl of 1 mg/ml of progesterone sulfate (18) (US, without phenol preservative, Eli Lilly Co.) and then incubated with [3H]estradiol, as described below.
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Fig. 1. Sucrose gradient centrifugation of cytoplasmic progesterone receptor in MCF-7 cells. Cells grown in 4 T-75 flasks were treated 4 days with MEM containing stripped calf serum, insulin, hydrocortisone, and prolactin, with (0) or without (X) 10 nM estradiol. Cytosols (6.1 and 5.1 mg of protein/ml, respectively) were incubated with 20 nM [3H]R5020 at 4° for 4 h, treated with DCC for 10 min, then centrifuged through 5 to 20% sucrose gradients prepared in phosphate buffer containing glycerol and thioglycerol. To determine nonspecific binding, cytosol from estradiol-treated cells was incubated with 20 nM [3H]R5020 plus 100-fold excess unlabeled R5020 (X). Arrow indicates 4.6 S sedimentation of [15C]bovine serum albumin.

Fig. 2. Growth and PgR in cells treated with Tamoxifen with and without addition of estradiol. Two days after plating cells were treated 5 days with MEM containing stripped calf serum, insulin, hydrocortisone, prolactin, and either 1 μM Tamoxifen alone (T) or together with 0.001 nm to 100 nm estradiol (E). Cells from four T-75 flasks per treatment group were pooled for assays. PgR was measured by single saturating dose assay: 200 μl of cytosol incubated 4 h at 4°, in triplicate, with 20 nM [3H]R5020 alone or with 100-fold excess R5020. After 15-min incubation with DCC suspension, cytosols were centrifuged and aliquots of supernatant counted to determine bound radioactivity. Data shown are corrected for nonspecific binding. Cytoplasmic and nuclear estrogen receptors were measured by the single saturating dose protamine assay described under "Experimental Procedures." Values have been corrected for nonspecific binding. Unoccupied cytoplasmic receptors (O, Rc, 4° incubation), unoccupied nuclear receptors (O, Rn, 4° incubation), occupied nuclear receptor (x, Rn-E, 30°-4° incubation), total cell receptors (Rc + Rn + Rn-E).

Estradiol is effective in displacing Tamoxifen from the estrogen receptor at doses 10-fold lower than the anti-estrogen dose because of its higher affinity for the ER binding site. While these studies suggest a role for ER in mediating the PgR response, direct studies on ER proved more conclusive.

Effects of Estradiol on PgR Expression and ER Distribution - Replicate flasks were grown 4 days on harvest medium containing 0.001 to 100 nm estradiol. Fig. 3 shows the effects of these doses on PgR induction and on the compartmentalization of ER at the end of 4 days. PgR was doubled by 0.001 nm estradiol and maximal induction (approximately 4-fold) was seen at 0.1 nm estradiol. This dose is near physiological, resembling circulating serum levels found in cycling adult women (22). Larger doses were no more effective in stimulating PgR.

We have also studied how PgR induction is correlated with effects on ER since our postulate that PgR identify ER-positive tumors that have remained hormone-dependent rests on the premise that PgR synthesis is controlled through an intact ER system. When grown on estrogen-free medium MCF-7 cells have unoccupied cytoplasmic (Rc) and nuclear (Rn) receptors.

Fig. 3. Effect of estradiol on estrogen receptor distribution and PgR levels in MCF-7 cells. Cells (four T-75 flasks per treatment group) were treated 4 days with increasing estradiol concentrations (0.001 to 100 nm) added to MEM containing stripped calf serum, insulin, hydrocortisone, and prolactin. Control flasks received the same medium without estradiol. PgR measured by single saturating dose assay: 200 μl of cytosol incubated 4 h at 4°, in triplicate, with 20 nM [3H]R5020 alone or with 100-fold excess R5020. After 15-min incubation with DCC suspension, cytosols were centrifuged and aliquots of supernatant counted to determine bound radioactivity. Data shown are corrected for nonspecific binding. Cytoplasmic and nuclear estrogen receptors were measured by the single saturating dose protamine assay described under "Experimental Procedures." Values have been corrected for nonspecific binding. Unoccupied cytoplasmic receptors (O, Rc, 4° incubation), unoccupied nuclear receptors (O, Rn, 4° incubation), occupied nuclear receptor (x, Rn-E, 30°-4° incubation), total cell receptors (Rc + Rn + Rn-E).

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In this study, the number of receptors in untreated cells was equally divided between these two compartments. With increasing doses of estradiol there is progressive depletion of Rc and Rn. At 0.1 nM only 15% of sites remain unfilled and virtually complete depletion occurs at higher doses. Rc does not remain in the cytoplasm in bound form (RcE, 30° incubation; not shown) or in cytoplasmic organelles (0.6 M KCl extract of high speed pellet). We conclude that Rc is translocated to the nucleus while Rn sites are filled so that all receptor is in the nucleus in bound form (RnE).

However, the quantity of RnE measured at different doses is puzzling. After 4-day treatment at the lower doses, although Rc and Rn decrease, there is little accumulation of occupied nuclear receptors (RnE). Instead, total cellular receptors (Rc + Rn + RnE) are progressively lower. At the higher estradiol doses free cytoplasmic and nuclear receptors are entirely depleted but total receptor levels (as RnE) are only 30% of total cell receptor present in controls. There appears to be a limit to the amount of receptor loss, however, so that at higher doses RnE accumulation occurs.

One interpretation of these effects is that at low doses all RnE formed from both Rc and Rn are rapidly utilized or processed in a subsequent step to a steady state level that is dependent on dose so that the number of RnE sites that can be processed reach saturation at approximately 0.1 nM estradiol. Excess RnE formed at higher doses remain unprocessed.

The extent of free receptor binding and processing parallels PgR introduction. Induction is incomplete at low doses if receptor binding and processing are incomplete, reaches a maximum at 0.1 nM when RnE processing is maximal, and is not increased further by accumulation of unprocessed RnE. This suggests that processing is an essential step in induction of a specific protein by estradiol.

**Kinetics of ER Processing**—The processed receptor levels seen in Fig. 3 were measured 4 days after the start of treatment. If processing is an essential step in genome activation, one would expect it to be an early event in estrogen action. Fig. 4A and B shows the effect of brief (10 min) or more prolonged (1 to 24 h) estrogen treatment. The dose used, 10 nM, is one at which processing capacity is saturated.

The untreated cells shown in Fig. 4A have 70% of free receptor in the nucleus and 30% in the cytoplasm. After 10 min on estradiol, Rc and Rn are no longer measurable and all cellular receptors appear in the nucleus bound to estradiol.

Despite the short incubation time (cells were treated, harvested, and cooled to 4° within 30 min), processing has started as shown by the decrease in total receptors in the estradiol-treated group. Panel B shows again that processing is well underway by 1 h so that maximal RnE buildup is not seen. Processing is essentially complete by 5 h thereafter, RnE is stabilized at the new steady state level (24 h and Fig. 5). We do not know whether this is a static state or a dynamic state involving continuous receptor turnover; the extended time course and replenishment studies below show that the cells are capable of new receptor synthesis.

**ER Distribution during Replenishment** —The compartmentalization of MCF-7 cells. A, effect of 10-min estradiol treatment. Cells were treated with harvest medium with (E) or without (C) 10 nM estradiol for 10 min, 37°, and then removed from flasks and cooled to 4° within 30 min), processing has started.

**ER during estrogen treatment and withdrawal.** Cells were treated continuously with 10 nM estradiol in harvest medium for the indicated times (+estradiol). After 4 days the hormone was removed from parallel replicate flasks (−estradiol) and, thereafter, cells were treated with harvest medium only. Control flasks (typical values shown at time 0) received only harvest medium throughout. Estrogen receptors determined by protamine sulfate assay. Rc, Rn, RnE, and total as in A. Each point represents triplicate determinations from four pooled T-75 flasks.

**FIG. 4.** Effect of short term estradiol treatment on ER distribution of MCF-7 cells. A, effect of 10-min estradiol treatment. Cells were treated with harvest medium with (E) or without (C) 10 nM estradiol, 37°, and then removed from flasks and cooled to 4° in 20 min. Cytoplasmic and nuclear ER were measured by the single saturating dose protamine exchange assay described under "Experimental Procedures." Uncoupled cytoplasmic receptors (Rc, 30°), unoccupied nuclear receptors (Rn, 4°), occupied nuclear receptors (RnE, 30°-4°), total cell receptors (Rc + Rn + RnE), B, cells treated as in A. Control cells receiving no estradiol are shown at 0 time. Replicate cells received estradiol for 1, 5, or 24 h and estrogen receptors were determined by protamine sulfate assay. Rc, Rn, RnE, and total as in A. Each point represents triplicate determinations from four pooled T-75 flasks.

**FIG. 5.** Subcellular distribution of ER during estrogen treatment and withdrawal. Cells were treated continuously with 10 nM estradiol in harvest medium for the indicated times (+estradiol). After 4 days the hormone was removed from parallel replicate flasks (−estradiol) and, thereafter, cells were treated with harvest medium only. Control flasks (typical values shown at time 0) received only harvest medium throughout. Estrogen receptors determined by protamine assay as described under "Experimental Procedures." Uncoupled cytoplasmic receptors (Rc, −−−), unoccupied nuclear receptors (Rn, O-O), occupied nuclear receptors (RnE, ×-×), total cell receptors (Rc + Rn + RnE, - - - ). Each point represents triplicate determinations from four pooled T-75 flasks.
eralization of ER following 12-day estrogen exposure or withdrawal is shown in Fig. 5. After estrogen treatment, Rc and Rn disappear, total receptor levels fall approximately 70%, and are found in the nucleus as RnE. Receptor levels then remain unchanged during the entire 12-day course of estrogen treatment. In cells from which estradiol has subsequently been removed, several effects are seen. The binding of estradiol to Rn (RnE) is remarkably prolonged. Although there is loss of RnE on Days 4 to 12, at least some estradiol always remains bound to nuclear receptor. Thus, restoration of cell ER cannot be explained by loss of estradiol from the nuclear receptor followed by redistribution of the newly emptied sites. Instead, both cytoplasmic receptors (Rc) and nuclear receptors (Rn) are clearly being synthesized de novo, and this synthesis is reflected in the restoration of total cellular ER. Since hormone withdrawal serves as a trigger of Rn reappearance, it seems unlikely that the nuclear localization of Rn is a result of translocation from the cytoplasm after estrogen binding.

Table I summarizes receptor distribution in untreated, processed, and replenished cells. In the latter, final Rc levels are below control while final Rn levels have returned to control. Totals are also the same as controls and the difference is in the high levels of RnE remaining in cells despite 8 days without estradiol.

**DISCUSSION**

We have shown that MCF-7 cells of human breast cancer origin respond to estradiol treatment with increased levels of PgR, as would be predicted from studies of chick oviduct (23), rat uterine PgR priming (24), and cyclic changes in PgR levels observed in the human endometrium (25). Our results clearly show that human breast cells which have undergone malignant transformation can continue to synthesize a specific protein under hormone control. Furthermore, these results lend credence to our hypothesis (5) that the presence of PgR in biopsies of human breast tumors indicates that in situ the tumor was exposed to and was capable of responding to circulating estrogens. Since the tumor, in one instance, has remained hormone responsive, one might suspect that other estrogen-responsive effects have also been retained.

We must emphasize, however, that PgR induction is only one product of estrogen action. The data cannot be construed to mean that other estrogen responses will necessarily be present. We find, for instance, that growth of MCF-7 cells is estrogen-sensitive, but unlike PgR, growth is not estrogen-dependent. Thus, effects of estrogens on growth and PgR induction might well be dissociated. We find this to be true in some dimethylbenz(a)anthracene tumors as well. Occasional tumors grow in castrate rats so that growth can be considered estrogen autonomous, while tumor PgR levels decline and could, therefore, be considered estrogen dependent (26). Conversely, we would predict that tumors regressing while on high dose estradiol would have elevated PgR levels.

Our studies strongly suggest that estrogen stimulation of PgR involves ER. First, the extent of PgR induction parallels closely both the binding and translocation of Rc and the binding of Rn. Second, PgR induction is correlated with ER processing during estradiol stimulation; when processing ceases PgR levels fall.

The nature of processing is unclear. It may be an active state in which a new equilibrium between degradation and synthesis is achieved (27, 28), or a redistribution of receptor within nuclear binding sites of differing affinities (29) or specificities (30), or sequestration of receptor to sites inaccessible to salt extraction (31, 32). Regardless, our data would suggest that the processing step is saturable, that peak activation occurs when RnE processing is maximal, and that the RnE accumulated in excess of that which is processed may be superfluous. That is, a dose of 0.1 nm estradiol is equal to 10 nm estradiol despite the fact that for the former some Rc...
remains, while for the latter, some unprocessed RnE remains. The ER processing seen in breast cancer cells may or may not be the same phenomenon observed in the rat uterus, where bound nuclear receptors are maximal after 1 h of estradiol treatment, with loss of 70 to 80% of sites by 6 h and complete loss by 24 h as cytoplasmic sites replenish (31, 33, 34). This may mean that without continuous stimulation, once activation occurs RnE function ceases so that it is degraded (35) and Rc resynthesized. However, it has also been proposed that continued binding and action of estrogens are required to elicit a sustained hormone response (33, 35). Similarly, the ability to stimulate uterine weight by antiestrogens or weak estrogens correlates with the time of nuclear receptor occupancy (35, 36). These would suggest that RnE processing is not the same phenomenon observed in the rat uterus, since the amount of receptor lost in processing is greater than 70 to 80% of sites by 6 h and complete loss by 24 h as cytoplasmic sites replenish (31, 33, 34). This may mean that without continuous stimulation, once activation occurs RnE function ceases so that it is degraded (35) and Rc resynthesized. However, it has also been proposed that continued binding and action of estrogens are required to elicit a sustained hormone response (33, 35). Similarly, the ability to stimulate uterine weight by antiestrogens or weak estrogens correlates with the time of nuclear receptor occupancy (35, 36). These would suggest that RnE processing is not simply a mechanism to terminate RnE action. Our studies show that during continuous estrogen treatment, processing suffices only in stabilizing RnE at a new steady state level and that continued stimulation may be required to activate the genome and maintain synthetic function.

Both Rc and Rn appear to be involved in estrogen action since the amount of receptor lost in processing is greater than can be accounted for by total loss of receptor from one of these compartments alone. Similarly, the restoration of receptors in both compartments upon estrogen withdrawal suggests that both receptors participate in estrogen action.

Most studies designed to show the effects of estrogens on subcellular ER distribution involve a single or pulsed dose of hormones (27, 31, 33, 34, 36) and show shifting receptor distributions during recovery from estrogen treatment. In vivo cells are almost never absolutely deprived of estrogen; instead, they are under continuous, albeit fluctuating, stimulation. Under such steady state conditions cytoplasmic and nuclear receptor levels represent the sum of receptor synthesis, translocation, and processing. This may be much lower than receptor levels potentially present in the unstimulated cell or which the cell is capable of synthesizing. It is such processed levels which are being measured in biopsied human tissues.

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Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor.
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