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MODULATION OF ESTROGEN RECEPTOR AND EPIDERMAL GROWTH FACTOR RECEPTOR mRNAs BY PHORBOL ESTER IN MCF 7 BREAST CANCER CELLS

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Previous studies have demonstrated an inverse relationship between estrogen receptor (ER) and epidermal growth factor receptor (EGF-R) gene expression in human breast cancer cells. This relationship was further investigated in MCF 7 cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). Exposure to 10 nM TPA resulted in a time-dependent increase in EGF-R mRNA, first apparent at 3 h and maximal between 9 and 24 h. There was a concomitant fall in ER mRNA with a maximum decline to 15-20% of control between 12 and 24 h. Although EGF-R mRNA levels declined between 24 and 72 h, both EGF-R mRNA and EGF-R binding remained above control levels and this was accompanied by a sustained depression of ER mRNA. These data support the view that ER and EGF-R gene expression is inversely regulated in human breast cancer and describe for the first time an inhibitory effect of a phorbol ester on steroid hormone receptor gene expression.

There are several studies documenting higher levels of EGF-R binding in biopsies of estrogen receptor negative (ER-) human breast cancer when compared with ER+ tumors (1-4). More recent studies have revealed that EGF-R mRNA levels are significantly higher in ER- compared with ER+ human breast cancer cell lines (5,6) and that there is a tight inverse relationship between the expression of these two receptor genes both within breast cancer cell lines as a whole and within an ER+ group (6). Such data raise the possibility that the expression of these genes is under reciprocal control either by the other gene product or by some common regulatory molecule. To further investigate this possibility we have initiated a series of studies monitoring the levels of ER and EGF-R mRNA following treatment with known regulators of ER and EGF-R concentrations. The present study addresses the effects of TPA which has been reported to induce EGF-R in some cell lines including human breast cancer cells (7,8).

Phorbol esters have diverse effects on cell proliferation and differentiation of cultured cells but are known to inhibit the proliferation of a number of breast cancer cell lines including MCF 7 (9-12). Part of the action of TPA appears to be due to its ability to activate protein kinase C leading to phosphorylation of the EGF-R and a resultant acute decrease in binding affinity. In addition to this well documented effect, TPA has recently been shown to increase EGF-R protein levels as a result of increased mRNA levels (7,8,12,13). Thus to further investigate the temporal relationship between EGF-R and ER

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gene expression we treated MCF 7 cells with TPA and monitored changes in ER mRNA and EGF-R mRNA by Northern blot analysis over a 72 h period.

MATERIALS AND METHODS

Materials. 12-O-tetradecanoylphorbol-13-acetate was obtained from the Sigma Chemical Co., St Louis, Missouri and stored as stock solutions (10^{-5} -10^{-2} M) in dimethylsulfoxide (DMSO) at -20°C. MCF 7 cells were supplied by E.G. and G. Mason Research Institute, Worcester, Massachusetts, for the National Cancer Institute Breast Cancer Program Cell Bank and maintained in culture as previously described (14). Receptor grade murine EGF was from Collaborative Research, Lexington, Massachusetts.

EGF Binding Studies. Receptor grade EGF was iodinated to a specific activity of 100-200 mCi/mg using a modified chloramine-T method as previously described (15). Cells were plated at a density of 10^5 cells/well into 24 well tissue culture trays in RPMI 1640 medium supplemented with: 20 mM Hepes buffer, 14 mM sodium bicarbonate, 6 mM l-glutamine, 20 μg/ml gentamicin, 10 μg/ml porcine insulin, 0.06% phenol red and 5% FCS and allowed to grow for 3 days at which time cell numbers had reached 6-8 x 10^5 cells/well. The culture medium was then poured off, the monolayer washed and the cells further incubated at 37°C with TPA or the vehicle (0.1% DMSO) in 1 ml of tissue culture medium containing 1% charcoal-treated FCS. After various periods of exposure to TPA the culture medium was poured off and the level of saturable EGF binding assessed by incubating with approximately 40,000 cpm ^{125}I-EGF (approximately 0.01 nM) in the presence or absence of 50 nM unlabelled EGF in 0.5 ml of binding buffer for 1 h at 37 C as previously described (15). Scatchard analysis was undertaken under identical conditions with EGF concentration in the range 0.01 - 10 nM.

ER and EGF-R cDNA clones. The 2.1 kb human ER cDNA, OR8 has previously been described in detail (16,17). The human EGF-R cDNA clone was a 1.8 kb fragment of the λ31 clone (18), previously described by Malden et al. (19). This cDNA clone encodes the extracellular EGF binding domain and the transmembrane domain of the EGF-R. Both cDNA inserts were excised from EcoR1 digested plasmid DNA and isolated by electrophoresis on 1.5% low-melting agarose gels.

Preparation of RNA and Northern Analysis. To study the effects of TPA on ER and EGF-R mRNA 1 x 10^6 exponentially growing MCF 7 cells were plated into replicate 150 cm^2 flasks in 50 ml of medium. Four days later the medium was changed to one containing 1% charcoal-treated FCS and TPA was added 24 h later. At varying times from 3-72 h, duplicate flasks were harvested, and the cells pelleted by centrifugation. RNA was extracted from these cells using the guanidinium isothiocyanate-cesium chloride method of Chirgwin et al. (20). Twenty μg RNA samples were size-fractionated on 1 % agarose-formaldehyde gels and transferred to zeta-probe nylon filters (Bio-Rad, Sydney, Australia) following the method of Maniatis et al. (21). Loading efficiency was monitored by ethidium bromide staining. The human ER and EGF-R cDNAs were nick translated to specific activities of 1-5 x 10^8 cpm/μg. Hybridization was carried out at 50°C for 16 h in a solution containing 50% (v/v) formamide, 2x SSPE (SSPE = 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 10% (w/v) dextran sulphate (molecular weight 500,000), 1% (w/v) SDS, 0.5% (w/v) Blotto (10% Diploma low fat dry milk powder, 0.2% azide), 40 μg/ml polyadenylic acid (5', Sigma), 0.2 mg/ml yeast RNA and 0.5 mg/ml heat denatured salmon testis DNA. Following hybridization, the filters were briefly rinsed in 2x SSC (SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), then washed in 2x SSC, 0.1% SDS at room temperature for 15 min with shaking, followed by preheated 0.2x SSC, 1% SDS at 65°C for 30 min with shaking and finally rinsed again in 0.5x SSC, 0.1% SDS. Autoradiography was carried out at -70° C using Kodak X-OMAT AR film. The autoradiographs were quantified using a Bio Rad Video Densitometer (Model 620) and the Bio Rad 1-D Analyst software for integration analysis.

RESULTS

Treatment of MCF 7 cells with TPA led to a concentration-dependent decrease in cell proliferation rate which was half-maximal at approximately 0.1 nM and maximal at 10 nM. The magnitude of this response was not increased further by increasing the TPA concentration to 1 μM (data not shown). Studies in other cell systems have demonstrated that sensitivity to TPA is associated with an acute decline in the ability of cells to bind EGF. This was confirmed with MCF 7 cells where the decreased growth rate was accompanied by a concentration-dependent decrease in specific EGF binding measured after 2 h exposure to TPA. This response was half-maximal at about 2.5 nM and maximal at 100 nM (data not shown). To further investigate the temporal changes in EGF-R binding following TPA treatment we monitored changes in specifically bound EGF over a 72 h period following administration of 10 nM TPA. There was an initial rapid decline in the ability of cells to bind EGF due to a marked decrease in the number of EGF-R sites (Fig. 1A). This decrease was maximal at 6 h. Thereafter the level of EGF-R increased rapidly and exceeded control levels by 15 h. By 24 h there was a 20-fold increase in EGF-R sites/cell but this was accompanied by a 7-fold decrease in receptor affinity (Fig. 1B).

In order to investigate this apparent increase in new protein synthesis we monitored the concentration of EGF-R mRNA over a 72 h time-course. A typical Northern blot is shown in Fig. 2 where it can be seen that untreated MCF 7 cells express extremely low levels of EGF-R mRNA which were undetectable at the exposure time used for this autoradiograph. Treatment with TPA resulted in a major increase in EGF-R mRNA at 6 h. The levels continued to increase and reached a maximum at 12 h which was maintained until 24 h before declining at 48 h and 72 h. However, at these later times a new steady state was maintained which was approximately 5-fold above control. A more detailed time course is presented in Fig. 3 where data from 2 separate experiments are pooled. These data reveal

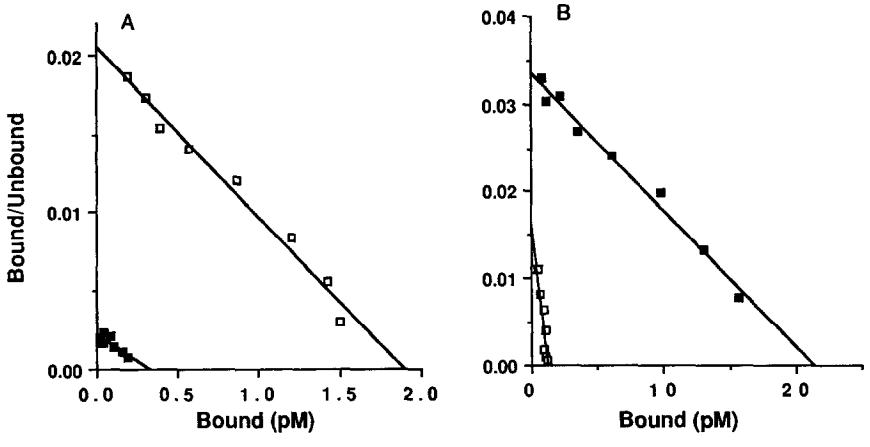

Figure 1. Effect of TPA on EGF binding to MCF 7 cells. MCF 7 cells were exposed to 10 nM TPA for 2 h (A) or 24 h (B) and then assessed for their ability to bind EGF according to the procedures outlined in Materials and Methods. Control = open symbols, TPA-treated = solid symbols.

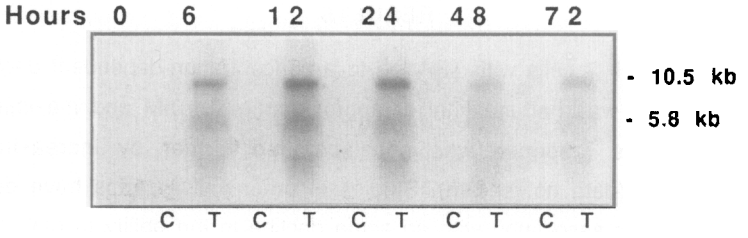

Figure 2. Effect of TPA treatment on EGF-R mRNA levels in MCF 7 cells. Cells were treated with 10 nM TPA and control (C) and TPA-treated (T) flasks harvested at 0, 6, 12, 24, 48 and 72 h. RNA was prepared and subjected to Northern analysis as described in Materials and Methods. The autoradiogram was developed after 22 h exposure with an intensifying screen.

that an approximate 5-fold increase in EGF-R mRNA was apparent as early as 3 h after TPA treatment. Furthermore presentation of the TPA-induced changes in EGF-R binding on the same graph illustrates the substantial lag between changes in EGF-R mRNA levels and changes in functional EGF-R, as assessed by whole cell radioligand binding.

Since we have previously demonstrated a tight inverse correlation between EGF-R and ER gene expression in a series of human breast cancer cell lines (6) the levels of ER mRNA were also measured following TPA treatment. A typical Northern blot of the effects of TPA on ER mRNA levels is shown in Fig. 4. These data illustrate the rapid depletion of the 6.4 kb ER mRNA following TPA treatment. The data from pooled experiments presented in Fig. 3 show that TPA treatment resulted in a rapid fall in ER mRNA; a 30% decline was apparent at 3 h with levels continuing to fall to a nadir of approximately 15% of control by 24 h. These depressed levels were maintained at a new steady state during the remainder of the experiment. Interestingly during the first 12 h of TPA treatment there was a tight inverse relationship between ER and EGF-R mRNA levels. Thereafter, however, ER mRNA levels remained constant in the face of declining but elevated levels of EGF-R mRNA and EGF-R binding.

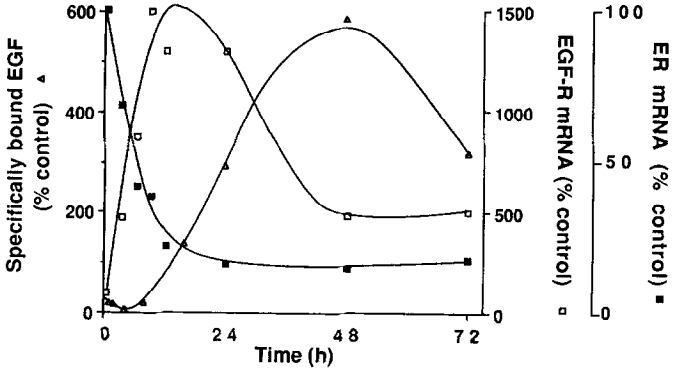

Figure 3. Temporal changes in EGF-R binding, EGF-R mRNA and ER mRNA levels following treatment of MCF 7 cells with TPA. Cells were treated with 10 nM TPA and at the times indicated the level of EGF-R binding (△) and the levels of EGF-R (□) and ER (■) mRNA were determined according to the procedures outlined in Materials and Methods. The data are the means of triplicate estimates (EGF-R binding) or duplicate experiments (EGF-R and ER mRNA).

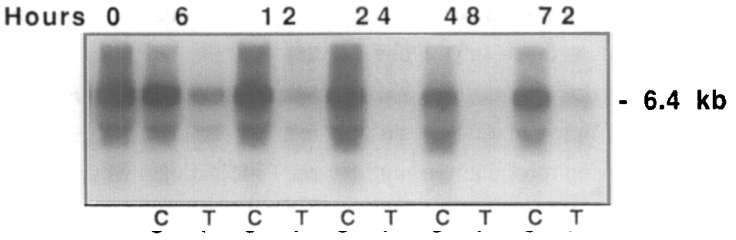

Figure 4. Effect of TPA treatment on ER mRNA levels in MCF 7 cells. Cells were treated with 10 nM TPA and control (C) and TPA-treated (T) flasks harvested at 0, 6, 12, 24, 48 and 72 h. RNA was prepared and subjected to Northern analysis as described in Materials and Methods. The autoradiogram was developed after 66 h exposure without an intensifying screen.

DISCUSSION

These data confirm earlier studies which demonstrated that TPA and other phorbol ester tumor promoters can inhibit cellular replication and saturable EGF binding in MCF 7 cells (9-11). In addition the current experiments provide novel information on: the long-term effects of TPA treatment on EGF-R binding in these cells, the temporal relationship between TPA-induced changes in EGF-R mRNA concentrations and total cellular EGF-R binding, and the relationship between EGF-R and ER gene expression following TPA treatment. The latter result which demonstrated a rapid depletion of ER mRNA following TPA treatment is, to our knowledge, the first demonstration of regulation of steroid hormone receptor gene expression by this class of compound and raises the exciting possibility of direct interactions between growth factor signal transduction pathways, particularly protein kinase C, and control of sensitivity to steroid hormones.

Treatment of various cell types with TPA is invariably associated with a rapid decline in EGF-R binding in sensitive cells and this was also apparent in the MCF 7 cells studied here. This phenomenon has previously been attributed to TPA activation of protein kinase C leading to phosphorylation of the EGF-R and a decrease in the affinity of the receptor for its ligand (22-24). Previous studies have demonstrated the ability of TPA to bind to protein kinase C, translocate the enzyme from a cytosol to a membrane compartment, and ultimately down-regulate protein kinase C levels in this cell line (25,26).

Following the acute decline in EGF-R binding during the first 6 h of TPA treatment EGF-R binding began to increase due to an increase in EGF-R number. However, there was a marked decline in binding affinity indicating that the newly synthesized receptor had lower affinity for the ligand. The increase in EGF-R concentration was preceded by a rapid and marked induction of EGF-R mRNA which had increased by 5-fold after 3 h with a maximal 15-fold increase at 9 h. These data are consistent with TPA having a direct effect on EGF-R gene expression in MCF 7 cells, a conclusion which is consistent with the reported TPA effects on EGF-R immunoreactive protein and mRNA levels in other cellular systems including MDA MB 468 breast cancer cells (7,8,13). Further support for a direct effect of TPA on EGF-R gene expression comes from data showing induction of EGF-R promoter activity following TPA treatment (27). The inability of TPA to down-regulate this newly

synthesized receptor is likely due to the dependence of this phenomenon on protein kinase C (13) which was depleted at these later times (25,26).

A major impetus for the current experiments was our previous observation of a tight inverse relationship between EGF-R and ER mRNA levels in a series of human breast cell lines (6) and following progestin treatment of T 47D cells where EGF-R mRNA is increased (15) and ER mRNA decreased (28) immediately following progestin administration. A similar inverse relationship was seen here during the first 12 h of TPA treatment and it is possible that this relationship occurred due to reciprocal regulation of these two genes via a phorbol ester response element. Evidence has recently been provided for increased EGF-R promoter activity following TPA treatment (27) but to date there are no data on the effects of phorbol esters on the ER promoter.

The inverse relationship between ER and EGF-R mRNA levels was less obvious beyond 24 h when ER mRNA levels remained depressed in the face of falling EGF-R mRNA concentrations. However, despite the fall in EGF-R mRNA and binding at later time points these parameters were maintained 3-6 fold above control, supporting the hypothesis that elevated EGF-R levels are associated with depressed ER concentrations in human breat cancer (1-6,28). A potential explanation for this effect is that elevated EGF-R acting via its second messenger pathways depresses ER gene transcription. There are precedents in the literature for decreased steroid receptor binding, which may be due to decreased mRNA, following treatment of breast cancer cells with polypeptide ligands acting via receptor tyrosine kinases e.g. insulin is known to depress ER concentrations (29) while EGF decreases PR levels (30).

In summary, these experiments provide further evidence that there is inverse regulation of ER and EGF-R gene expression in human breast cancer cells. Such data imply that regulation of sensitivity to mitogenic growth factors and steroid mitogens may be closely linked in some steroid responsive cells. A more detailed understanding of the control of steroid hormone receptor gene expression by polypeptide hormones and growth factors is critical to a deeper knowledge of growth control in human breast cancer.

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