Effect of Cadmium on Estrogen Receptor Levels and Estrogen-induced Responses in Human Breast Cancer Cells*

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The effects of cadmium on estrogen receptor and other estrogen-regulated genes in the human breast cancer cell line MCF-7 were studied. Treatment of MCF-7 cells with 1 μM cadmium decreased the level of estrogen receptor 58%. Cadmium induced a parallel decrease in estrogen receptor mRNA (62%). Progesterone receptor levels increased 3.2-fold after cadmium treatment. This induction was blocked by the anti-estrogen ICI-164,384. Progesterone receptor mRNA was also increased by cadmium, as well as cathepsin D mRNA. An in vitro nuclear transcription run-on assay showed that cadmium increased the transcription of the progesterone receptor and pS2 genes and decreased transcription of the estrogen receptor gene. These are not general effects of heavy metals, as zinc, 25 and 100 μM, did not affect progesterone receptor protein and mRNA levels. Cadmium stimulated pS2 and progesterone receptor mRNAs in a clone of MDA-MB-231 cells transfected with the human estrogen receptor, but had no effect in MDA-MB-231 cells transfected with antisense estrogen receptor. Cadmium also stimulated an estrogen response element in transient transfection experiments. These data suggest that the effects of cadmium are mediated by the estrogen receptor independent of estradiol. In addition to its effect on gene expression, cadmium induced the growth of MCF-7 cells 5.6-fold.

Several reports demonstrate an effect of heavy metals on the structure and function of steroid receptors. Cadmium and selenium inhibit ligand binding to the glucocorticoid receptor, whereas zinc has no effect (3). Fishman et al. (4) have reported an increase in specific estradiol binding in rat uterine cytosol in response to copper treatment. A recent report by Predki et al. (5) demonstrates that zinc in the zinc fingers of the estrogen receptor can be replaced by several other metals such as copper, cadmium, nickel, and cobalt. The replacement of zinc by cadmium and cobalt has no effect on DNA binding, whereas replacement by nickel and copper inhibits ER binding to its response element.

Heavy metals have also been shown to have specific effects on several other biological systems. Cadmium may affect signal transduction pathways. For example, it has been shown that cadmium induces inositol polyphosphate formation and increases the level of cytosolic-free calcium in a variety of cell types (6). Calcium channels are also permeable to cadmium in different cell types (7, 8). Blockade of calcium channels by cadmium has also been reported (9–12). Induction of the expression of several different genes in response to heavy metals has also been reported. Zinc and cadmium induce the mRNA levels for 12-O-tetradecanoylphorbol-13-acetate-inducible genes in Swiss 3T3 cells (13). c-jun and c-myc mRNAs also increase after cadmium treatment in rat L6 myoblasts (14), and c-myc mRNA is also induced by cadmium in NK-49F cells (15). Cadmium also increases transcription of the heme oxygenase gene in mouse hepatoma cells (16). The expression of the 70-kDa heat shock protein in a rainbow trout hepatoma cell line increases in response to zinc; in addition, the same protein from chinook salmon embryonic cells is induced by zinc and cadmium (17).

Regulation of expression and activity of estrogen receptor plays an essential role in the growth, differentiation, and prognosis of human breast cancer. After binding to its cognate ligand, the ER becomes activated and stimulates transcription of several estrogen-inducible genes. In this study we demonstrate an effect of the heavy metal cadmium on ER levels and activity in human breast cancer cells. We measured the effects of cadmium on several estradiol-induced responses such as progesterone receptor (PgR), cathepsin D and pS2 induction, as well as its growth effects. Cadmium has an estrogen mimetic effect which cannot be extended to other heavy metals. The effects of cadmium appear to be mediated by the ER independent of estrogen binding.

MATERIAL AND METHODS

Tissue Culture—Wild type MCF-7 human breast cancer cells were grown in improved minimal essential medium (IMEM) supplemented with 5% fetal calf serum (FCS). At 70% confluence, the media were

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† The abbreviations used are: ER, estrogen receptor; IMEM, improved minimal essential medium; FCS, fetal calf serum; CCS, charcoal-stripped calf serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pairs; CAT, chloramphenicol acetyltransferase; ERE, estrogen response element.
changed to phenol-red IMEM supplemented with 5% charcoal-stripped calf serum (CCS). Cells were maintained in this media for 3 days prior to treatments. A clonal population derived from MCF-7 breast cancer cells (E3 clone) was provided by Dr. S. Brooks, Michigan Cancer Foundation, Detroit, MI. Cells were maintained in Dulbecco's modified Eagle's medium and Ham's F-12 medium 1:1 (v:v) supplemented with 5% FCS, 20 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin. Medium was changed to phenol-red IMEM supplemented with 5% CCS for 1 week before treatment. Two different cell lines derived from MDA-MB-231, S-30, and AS-23 cell lines (18) were provided by Dr. V. Craig Jordan, Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, Madison, WI. Cells were grown in phenol-red IMEM supplemented with 5% CCS, 26.2 mM HEPES, 1 μM insulin, and 400 μg/ml G418. Cells were treated with CdCl₂ (Sigma), ZnSO₄ (Sigma), estradiol, or the steroid antiestrogen ICI-164,384 (N-n-butyl-N-methyl-11-(3,17-dihydroxyoestr-1,3,5(10)-trien-7-yl)undecamid) (ICI Pharmaceuticals).

Measurement of ER and PgR Protein Levels—Cells were grown as described above. After 24-h treatment, the cells were washed twice with phosphate-buffered saline and pelleted by centrifugation. Cell pellets were sonicated in a high salt buffer (19), and the homogenate was incubated on ice for 30 min and centrifuged at 100,000 × g for 1 h at 4 °C. Supernatants were assayed for ER and PgR protein. The levels of ER and PgR protein were determined using specific enzyme immunoassay kits from Abbott Laboratories (North Chicago, IL). Aliquots of the total extracts were analyzed according to the manufacturer's instructions.

Measurement of ER and PgR mRNA Levels—Total cellular RNA was extracted from cells as described previously (19). The level of ER and PgR mRNAs was determined by a RNase protection assay. 32P-Labeled antisense RNA (cRNA) was synthesized in vitro from pER300 (estrogen receptor) (19), p36B4 (19), pGAPDH (glyceraldehyde-3-phosphate dehydrogenase) (20), and pS2 (21) using T7 polymerase and from pPgR250 (progesterone receptor) using SP6 polymerase. The pPgR250 riboprobe is a 250-bp AcaI fragment of PgR cDNA subcloned into pGEM 4. Sixty μg of total RNA were hybridized for 16 h to the 32P-labeled cRNAs. The samples were then digested with RNase A for 30 min at 25 °C. The protected cRNA probes were resolved on 6% polyacrylamide gels. The bands were visualized by autoradiography and quantified by optical densitometry. The levels of ER and PgR mRNAs were normalized using 36B4 and GAPDH as internal controls respectively.

Measurement of Cathepsin D mRNA Levels—Total RNA was isolated from MCF-7 cells as described previously (22). 20 μg of total RNA was electrophoresed on a denaturing 1.2% agarose, 2.2 M formaldehyde gel. The gel was stained with ethidium bromide to show RNA integrity. The RNA was transferred to Biotrans nylon membranes (ICN Biomedicals, Costa Mesa, CA) and hybridized to either a 2.0-kilobase pair human EcoRI-EcoRI 32P-labeled nick-translated cathepsin D cDNA probe kindly provided by Dr. Henri Rochefort, Montpellier, France (23) or to a 770-bp human β-actin cDNA probe (Oncor, Gaithersburg, MD) as described previously (24).

Transcription Elongation—To determine the level of gene transcription, an in vitro transcription run on assay was performed. Nuclei were isolated as described previously (25). Elongation of newly synthesized transcripts was performed in the presence of [32P]UTP. The radiolabeled RNA transcripts were isolated and hybridized to denatured plasmid DNA immobilized on a nitrocellulose filter. The denatured plasmids used for detection of specific transcripts were Q7 (ER), 36B4, PS2, and PGR as described previously (19). Autoradiographs were analyzed by densitometry, and the level of transcription of ER, PS2, and PGR was normalized to the transcription of the 36B4 gene.

Anchorage-dependent Growth Assays—MCF-7 cells were plated at 10⁵ cells/well into 12-well cluster dishes in IMEM supplemented with 5% FCS. Cells were grown to 40% confluence, and the medium was changed to phenol-red IMEM supplemented with 5% CCS. After 2 days in this media, cells were treated with either 1 nM estradiol or 1 μM cadmium chloride. Media, with the appropriate treatments, were replaced every 2 days. Cells were trypsinized at the specific time points and counted with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

Transient Transfection Assays—MCF-7 cells were plated at 5 x 10⁴ cells/dish into 100 x 20-mm tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) in IMEM supplemented with 10% FCS. 24 h after plating, cells were transiently transfected with 30 μg of the Vit-TR-CAT plasmid (26) using the low temperature, low CO₂ method (27). After 24 h, medium was changed to IMEM phenol-red free for 2 days, and cells were treated with either 1 nM estradiol or 1 μM cadmium chloride. The level of CAT protein was determined with an enzyme-linked immunosorbent assay kit (Boehringer Mannheim).

RESULTS

Effect of Cadmium Treatment on the Level of ER Protein—The level of ER protein in MCF cells after 24-h cadmium treatment was determined by an enzyme immunoassay. The data are represented as femtomoles of ER/mg of protein (Fig. 1A). A dramatic decrease in the protein level of ER was detected after a 24-h treatment with 1 μM cadmium. The ER level decreased from 282.4 fmol/mg of protein in control cells to 294.7 fmol/mg of protein in cadmium-treated cells. As expected from previous data obtained in this laboratory (19), 1 nM estradiol also produced a comparable decrease in ER protein levels. A similar decrease was observed when cells were treated with cadmium plus estradiol. Similar results were obtained when a clonal population derived from MCF-7 cells (E3 clone), the ER levels decreased from 282.4 fmol/mg of protein in control cells to 114.4 fmol/mg of protein in cadmium-treated cells (data not shown).

Effect of Cadmium Treatment on the Steady State Level of ER mRNA—An RNase protection assay was performed to determine the effect of cadmium on ER mRNA levels. 60 μg of total RNA were hybridized with a 32P-labeled antisense riboprobe for...
Cadmium Regulation of ER Levels and Activity

Effect of Cadmium Treatment on the Steady State Level of PgR mRNA—To determine the effect of cadmium on PgR mRNA, MCF-7 cells were treated with 1 µM cadmium for 24 h, and the level of PgR mRNA was measured by an RNase protection assay. Fig. 2B shows a typical autoradiograph where the 250- and 104-bp bands represent the protected fragments for PgR and GAPDH, respectively. GAPDH is used as internal control, since its expression is not affected by estradiol. The basal level of PgR mRNA in MCF-7 cells was undetectable in control estradiol-depleted cells, whereas 1 µM cadmium induced PgR mRNA to levels easily detected after short exposure of the gel to x-ray film. An increase in PgR mRNA was also observed when cells were treated with 1 nM estradiol. Since the mRNA for PgR in control cells was not detectable, it is not possible to determine the fold increase in PgR mRNA by either cadmium or estradiol. To determine if the effects of cadmium and estradiol on PgR mRNA were of the same relative magnitude, the bands for PgR were quantified by scanning densitometry and normalized to the level of GAPDH. Following treatment with cadmium, the ratio of the PgR signal to the GAPDH signal was 0.38 (±0.27). Following treatment with estradiol, the ratio of the signal of PgR to GAPDH was 0.72 (±0.08). The effect of cadmium on PgR mRNA levels appears to be comparable with the effect of estradiol on PgR mRNA and to parallel its effect on PgR protein levels.

To determine if the effect of cadmium on PgR could be extended to another estrogen regulated gene, a Northern blot hybridization was performed to ascertain the effect of the heavy metal on the mRNA levels of cathepsin D, a well known estrogen-responsive gene (23). Cadmium, 1 µM, significantly induced the expression of the 2.2-kilobase mRNA for cathepsin D (data not shown). Estradiol, 1 nM, also induced the expression of cathepsin D to a similar level.

Effect of Cadmium Treatment on Gene Transcription—To determine if the effect of cadmium on ER and estrogen-regulated genes occurs at the transcriptional level, in vitro nuclear transcription run-on experiments were performed. Nuclei were isolated from cells treated with cadmium, 1 µM, and transcription elongation in isolated nuclei was performed in the presence of [32P]UTP. The newly synthesized transcripts were isolated and hybridized to cDNA probes for the specific genes of interest. Cadmium regulation of PgR and pS2 transcription was studied, since the transcription of both genes is stimulated by estradiol (28, 29). The level of transcription of each gene was normalized to the level of transcription of 36B4. The transcription of the ER gene decreased after cadmium treatment with a 60% inhibition observed after 6 h of cadmium treatment (Fig. 3A). Cadmium significantly increased the transcription of PgR by 12-fold and pS2 by 7-fold (Fig. 3B). In both cases, 1-h treatment with cadmium was sufficient to observe a significant increase in transcription.

Effect of Cadmium on the Growth of MCF-7 Cells—The effect of 1 µM cadmium on the anchorage-dependent growth of MCF-7 cells was also studied. As shown in Fig. 4, cadmium significantly stimulated the growth of MCF-7 cells when compared with cells grown in estrogen-depleted medium to a level that was comparable with the degree of growth stimulation induced by estradiol. The effect of estradiol and cadmium on cell growth does not appear to be additive.

Dependence of the Cadmium Effect on the Presence of Functional Estrogen Receptor—Several experimental approaches were undertaken to determine if the effect of cadmium was mediated by the ER. In the first approach, the effect of the anti-estrogen ICI-164,384 was tested in the MCF-7 cloned cell line E3. ICI-164,384 (100 nM) had no effect on PgR protein levels. Following treatment with the anti-estrogen, the level of PgR was 9 fmol/mg of protein when compared with control
values of 11 fmol PgR/mg of protein. Cadmium treatment resulted in an increase in PgR levels to 145 fmol/mg of protein. When MCF-7 cells were incubated with cadmium and the anti-estrogen, the PgR protein levels were similar to control levels, 8 fmol/mg of protein, demonstrating that the anti-estrogen completely inhibited the cadmium-induced increase in PgR.

In the second experimental approach, the effect of cadmium on PgR and pS2 mRNAs was tested in S-30 breast cancer cells. These cells are a clone of MDA MB-231, an ER-negative breast cancer cell line which has been transfected with the human estrogen receptor (18). In the same experiment, the effect of cadmium on AS-23 breast cancer cells was also tested. This cell line is also a clonal derivative of MDA MB-231 cells, but has been transfected with an antisense ER expression vector. As shown in Fig. 5, cadmium induced the pS2 mRNA in S-30 cells, but had no effect on pS2 mRNA expression in the AS-23 cells. The same results were obtained for PgR mRNA (data not shown). These results suggest that cadmium activates estrogen-regulated genes through the estrogen receptor.

Effect of Zinc Treatment on the Steady State Level of PgR Protein.—To ascertain if the effects of cadmium could be observed with other heavy metals, the effect of zinc on PgR protein and mRNA levels in MCF-7 cells was determined. Zinc, 25 μM, had no significant effect on PgR protein levels and was unable to block the effect of cadmium. Following treatment with zinc, the level of PgR was 9 fmol/mg of protein when compared with control values of 11 fmol PgR/mg of protein. Cadmium treatment resulted in an increase in PgR levels to 145 fmol/mg of protein. When MCF-7 cells were incubated with cadmium and zinc, the PgR protein levels were similar to levels with cadmium alone, 140 fmol/mg of protein. Zinc, 25 or 100 μM, also failed to have an effect on PgR mRNA levels (Fig. 6). These results suggest that the effects observed with cadmium are specific and not a general property of other heavy metals.

Cadmium Induction through an Estrogen Response Element (ERE)—To determine whether cadmium modulated ER activity through an ERE, a transient transfection assay was employed. MCF-7 cells were transfected with the plasmid Vit-TK-CAT. This plasmid contains the chloramphenicol acetyltransferase gene under the transcriptional control of a thymidine kinase promoter and the ERE derived from the vitellogenin gene (25). The level of CAT was determined with an enzyme-linked immunosorbent assay kit. The experiments were repeated twice, and the results of a typical experiment are shown in Fig. 7. Cadmium, 1 μM, as well as estradiol, stimulated the expression of CAT by approximately 2-fold; cadmium plus estradiol had the same effect as either agent alone. These results suggest that the effects of cadmium are mediated by the estrogen receptor through an ERE.

**DISCUSSION**

The purpose of this study was to determine the effect of cadmium on ER expression and on other estrogen-induced re-
Cadmium Regulation of ER Levels and Activity

Fig. 6. Effect of zinc on the PgR mRNA levels. MCF-7 cells were grown as described in the legend to Fig. 1. After treatment with estradiol and zinc, 25 and 100 µM, the levels of PgR mRNA were determined by RNase protection. Lane 1, control; lane 2, 1 nM estradiol; lane 3, 1 µM zinc; lane 4, 25 µM zinc; lane 5, cadmium + 25 µM zinc; lane 6, 100 µM zinc; lane 7, cadmium + 100 µM zinc.

Fig. 7. Effect of cadmium on ERE activity. MCF-7 cells were transiently transfected with the reporter gene Vit-TK-CAT as described under "Material and Methods." The expression of the CAT protein was determined by enzyme-linked immunosorbent assay.

eresponses. We have examined the cadmium effects on ER protein and mRNA levels as well as on ER gene transcription. The effects of cadmium were also evaluated on several other ER-regulated genes. Our results indicate that cadmium has a significant effect on ER expression and activity. Cadmium mimics the effects of estradiol. Like the hormone, cadmium decreases the steady state levels of ER protein and mRNA. Previous work from this laboratory showed a similar quantitative effect of estradiol on ER protein and mRNA levels in MCF-7 cells (19).

The effect of cadmium on ER expression occurs at the transcriptional level, as evidenced by an inhibition of ER gene transcription. In order to determine if cadmium also mimics other estrogenic responses in MCF-7 cells, several estrogen-regulated genes and responses were also studied. PgR protein and mRNA levels significantly increased following a 24-h treatment with 1 µM cadmium. The same inductive effect by cadmium was also observed in the ER-negative breast cancer cell line (MCF-7 cells), transfected with the estrogen receptor (S-30 MB-231 cells), transfected with the estrogen receptor (S-30 cells) (18), was treated with cadmium, it exhibited an increase in the mRNA transcripts for PgR. Experiments done in the same parental cell line but transfected with the antisense for ER (AS-23 cells) did not show any cadmium effect on the transcription of the PgR.

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etiology and/or progression of breast cancer. Although exposure to cadmium is primarily associated with cardiovascular disease, several reports show an association of cadmium exposure with prostate cancer (41–44). To date there are no studies of cadmium and breast cancer. The cadmium effects on biological systems are of particular interest, since cadmium is routinely used to induce the estrogen receptor pathway responsible for at least part of the observed results. Additional experiments are required to understand the mechanism of the cadmium effect on estrogen receptor expression.

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