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Estrogen-Like Activity of Metals in Mcf-7 Breast Cancer Cells

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The ability of metals to activate estrogen receptor-α (ERα) was measured in the human breast cancer cell line, MCF-7. Similar to estradiol, treatment of cells with the divalent metals copper, cobalt, nickel, lead, mercury, tin, and chromium or with the metal anion vanadate stimulated cell proliferation; by d 6, there was a 2- to 5-fold increase in cell number. The metals also decreased the concentration of ERα protein and mRNA by 40–60% and induced expression of the estrogen-regulated genes progesterone receptor and pS2 by1.6- to 4-fold. Furthermore, there was a 2- to 4-fold increase in chloramphenicol acetyltransferase activity after treatment with the metals in COS-1 cells transiently cotransfected with the wild-type receptor and an estrogen-responsive chloramphenicol acetyltransferase reporter gene. The ability of the metals to alter gene expression was blocked by an antiestrogen, suggesting that the activity of these compounds is mediated by ERα. In binding assays the metals blocked the binding of estradiol to the receptor without altering the apparent binding affinity of the hormone ($K_d = 10^{-10}$ M). Scatchard analysis employing either recombinant ERα or extracts from MCF-7 cells demonstrated that ^{57}Co and ^{63}Ni bind to ERα with equilibrium dissociation constants of 3 and 9.5×10^{-9} and 2 and 7×10^{-9} M, respectively. The ability of the metals to activate a chimeric receptor containing the hormone-binding domain of ERα suggests that their effects are mediated through the hormone-binding domain. Mutational analysis identified amino acids C381, C447, E523, H524, N532, and D538 as potential interaction sites, suggesting that divalent metals and metal anions activate ERα through the formation of a complex within the hormone-binding domain of the receptor. (*Endocrinology* 144: 2425–2436, 2003)

ESTROGENS are a family of steroidal hormones that are synthesized in a variety of tissues, but are produced primarily in the ovaries during reproductive years. One of the main functions of estrogens is to promote the growth and differentiation of sexual organs and other tissues related to reproduction. The disruption of the reproductive system of male and female animals in the wild has been attributed to exposure to environmental contaminants that mimic the effects of estradiol. It has been suggested that the high incidence of hormone-related cancers and diseases in the Western world is also due to the presence of environmental estrogens (1). In fact, a number of chemicals in the environment demonstrate estrogen-like activity when tested in biological systems (reviewed in Ref. 2). Phytoestrogens, such as coumestrol and the isoflavone genistein, are naturally occurring chemicals with estrogen-like activity, whereas xenoestrogens are synthetic chemical contaminants in the environment. Although xenobiotics mimic the effects of estrogens, most lack the phenathrene ring structure of estradiol. In fact, the xenoestrogens represent a structurally diverse group of hydrocarbons. Although there is no common structural motif, many xenoestrogens contain one or two aromatic rings. In addition, some xenoestrogens are chlorinated, giving them a negative charge. Bisphenol A is a xenobiotic that was originally synthesized for pharmaceutical use as a synthetic estrogen, but was later used as a cross-linking reagent in the production of plastic. However, most xenoestrogens were not designed to activate the estrogen receptors (ERs). Organochlorides, such as dicholorodiphenyltrichloroethane and kepone, were designed for use as pesticides, whereas alkylphenols are used in the manufacture of plastics and detergents and as antioxidants. In addition to hydrocarbons and organochlorides, recent studies suggest that metals may represent a new class of endocrine disrupters (3–6). Cadmium, selenite, and arsenite have been shown to activate ERα by a mechanism involving the hormone-binding domain of the receptor. The present study provides evidence that several additional metals, including cobalt, copper, nickel, chromium, lead, mercury, tin, and vanadate, may be members of this class of endocrine disrupters.

The ER is a known metal-binding protein. The binding of zinc to cysteines in the DNA-binding domain of ERα results in the formation of a protein motif referred to as a zinc finger (7). Other metals have been shown to substitute for zinc in the zinc finger of ERα and to alter the ability of the DNA-binding domain to bind to an estrogen response element (8). The replacement of zinc with either nickel or copper inhibits the binding of the DNA-binding domain to an estrogen response element, whereas replacement of zinc with either cadmium or cobalt has no effect on binding. In addition to

Abbreviations: CAT, Chloramphenicol acetyltransferase; CCS, charcoal-stripped calf serum; ER, estrogen receptor; FCS, fetal calf serum; IMEM, improved minimal essential medium; RNase, ribonuclease.

the DNA-binding domain, metals have been shown to bind to the hormone-binding domain of the receptor and in some cases to block the binding of estradiol. Zinc, nickel, and cobalt have been shown to interact with the hormone-binding domain of ER immunopurified from calf uterus and noncompetitively block the binding of estradiol (9), whereas copper and lead decrease the binding of estradiol to the ER in human, rabbit, and rat uterus (10–12). In the case of copper, the ability of the metal to inhibit ligand binding is mediated by cysteines and histidines located in the hormone-binding domain of the receptor (11). Previous studies from this laboratory have also shown that cadmium, arsenite, and selenite bind to the hormone-binding domain of ERα and inhibit the binding of estradiol (4–6). More important than their ability to interfere with ligand binding, these studies demonstrated that cadmium, arsenite, and selenite activate the ER through a high affinity interaction with the hormone-binding domain of the receptor (3–6). The results in the present study show that copper, cobalt, nickel, lead, mercury, tin, chromium, and vanadate also activate ERα, suggesting that metals and metalloids may constitute a new class of nonsteroidal environmental estrogens.

To determine whether other metals also have estrogen-like properties, the effects of cobalt, copper, nickel, chromium, lead, mercury, tin, and vanadate on ERα expression and activity were studied in the ER-positive breast cancer cell line, MCF-7. All of these compounds induced the estrogen-regulated genes progesterone receptor and pS2 and activated ERα through an interaction with the hormone-binding domain, an interaction that also blocked estradiol binding to the receptor. The interaction of the metals with ERα involves several amino acids in the hormone-binding domain, suggesting that the metals may form a high affinity complex with the hormone-binding domain and thereby activate ERα.

Materials and Methods

Tissue culture

The human breast cancer cells, MCF-7, were grown in improved minimal essential medium (IMEM) supplemented with 5% fetal calf serum (FCS). At 80% confluence, the medium was changed to phenol red-free IMEM supplemented with 5% charcoal-stripped calf serum (CCS). Cells were maintained in this medium for 2 d before treatment. Cells were treated with cobalt (II) chloride (Co), cupric chloride (Cu), nickel (II) chloride, sodium metavanadate (V), mercuric chloride (Hg), lead (II) chloride, tin (II) chloride (Sn), chromium (II) chloride (Cr), chromium (III) chloride (Cr^{III}), zinc sulfate (Zn), estradiol (Sigma Aldrich Corp., St. Louis, MO), or the steroidal antiestrogen ICI-182,780 (*N*-*n*-butyl-*N*-methyl-11(3,17β-dihydroxyoestra-1,3,5-(10)-trien-7α-yl)undecamide; Zeneca Pharmaceuticals, Wilmington, DE).

Anchorage-dependent growth assays

MCF-7 cells were plated at 10^5 cells/well into six-well plates in IMEM supplemented with 5% FCS. Cells were grown to 40% confluence, and the medium was changed to phenol red-free IMEM supplemented with 5% CCS. After 2 d in this medium, cells were treated with either 10^{-9} M estradiol or 10^{-6} M metal salt. Medium with the appropriate treatments was replaced every 2 d. Cells were trypsinized at the specific time points and counted with a Coulter counter (Coulter Electronics, Inc., Hialeah, FL).

Measurement of ER and progesterone receptor protein concentrations

Cells were grown as described above. After a 24-h treatment, cells were washed twice with PBS and pelleted by centrifugation. Cell pellets were sonicated in a high salt buffer (13), 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 ERα and progesterone receptor protein. The concentrations of ERα and progesterone receptor 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, progesterone receptor, and pS2 mRNA

Total cellular RNA was extracted from cells as described previously (13). The amounts of ERα, progesterone receptor, and pS2 mRNA were determined by ribonuclease (RNase) protection assay. ^{32}P-Labeled antisense RNA (cRNA) was synthesized *in vitro* from pOR300 (ERα) (13), acidic ribosomal phosphoprotein PO (36B4) (13), pS2 (14), and glyceraldehyde-3-phosphate dehydrogenase (15) using T7 polymerase and from pPgR250 (progesterone receptor) using SP6 polymerase (13). Sixty micrograms of total RNA were hybridized for 16 h to the ^{32}P-labeled cRNAs. The protected cRNA probes were resolved on 6% polyacrylamide gels. The bands were visualized by autoradiography and quantified by phosphorimaging. The amounts of ERα and pS2 mRNA were normalized using 36B4 as an internal control. The amounts of progesterone receptor mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase.

Transient transfection assays

The Lipofectamine Plus (Life Technologies, Inc./BRL, Gaithersburg, MD) method was employed to transfect COS-1 cells. COS-1 cells were plated at a density of 1×10^6 cells/100-mm dish in phenol red-free IMEM containing 10% CCS for 24 h. The cells were transfected with 5.7 μg DNA containing 0.5 μg of an ERα expression vector (wild-type or mutant, as described below), 5 μg of a reporter construct, and 0.2 μg β-galactosidase or *Renilla* (Promega, Madison, WI) according to the manufacturer's instructions. Sixteen to 18 h after transfection, cells were replenished with phenol red-free IMEM containing 10% CCS in the presence or absence of 10^{-9} M estradiol or 10^{-6} M metal salt. The cells were harvested 24 h later, and chloramphenicol acetyltransferase (CAT) or luciferase activity was measured as described previously (3). CAT activity was expressed as the percent conversion of chloramphenicol to its acetylated forms and was normalized to the activity of β-galactosidase. Luciferase was normalized to the activity of *Renilla*. The increase in CAT and luciferase activity in response to treatment is expressed relative to untreated controls.

Expression vectors for the wild-type ERα, the amino acid mutants (C381A, C417A, C447A, C530A, E523Q, D538N, H524A, K529Q, K531Q, and N532D), and the helix H12 mutants (ERTAF-1 and ER-null) were described previously (16–20). For these transient transfection assays, the estrogen-responsive reporter construct pbCAT-(S)MERE was used (21). The chimeric receptor GAL-ER and the reporter plasmid 17M2GCAT were also described previously (22).

ER binding assays

The ability of the metal to block estradiol binding to ERα was determined in cell extracts from MCF-7 cells maintained in phenol red-free IMEM containing 5% CCS. After 2 d in estrogen-depleted medium, the cells were lysed by sonication in a high salt buffer containing 10 mM Tris (pH 7.5), 1.5 mM EDTA, 5 mM sodium molybdate, 0.4 M KCl, 1 mM monothioglycerol, and 2 mM leupeptin. The homogenate was incubated on ice for 30 min and centrifuged at 100,000 × *g* for 1 h at 4 C (4). The protein concentration of the cell extract was determined by the Bradford method. Cell extracts were preincubated with 10^{-6} M metal salt. [^{3}H]Estradiol (10^{-12}–10^{-7} M) was then added in the presence and absence of a 200-fold molar excess of diethylstilbestrol and incubated at 37 C for 2 h. Free steroid was removed by the addition of 5% dextran-coated charcoal. The amount of radioactivity was measured by scintillation counting. The data were analyzed by the method of Scatchard (23).

The ability of the metals to block estradiol binding was also tested

using recombinant human ERα (PanVera Corp., Madison, WI). Recombinant ERα (4×10^{-9} M) was preincubated for 1 h on ice with 10^{-6} M metal salt. [^{3}H]Estradiol (10^{-8} M) was then added in the presence and absence of a 200-fold molar excess of diethylstilbestrol and incubated at 37 C for 2 h. Free steroid was removed by the addition of 5% dextran-coated charcoal. The amount of radioactivity was measured by scintillation counting. Specifically bound complexes were calculated by subtracting nonspecific binding from total binding.

The ability of radioactive cobalt and nickel to bind ERα was determined using recombinant ERα and MCF-7 extracts. Recombinant ERα (4×10^{-9} M) was incubated with various concentrations of ^{63}Ni and ^{57}Co [10^{-12}–10^{-7} M; specific activity of ^{63}Ni, 9.78 mCi/mg (NEN Life Science Products, Boston, MA); specific activity of ^{57}Co, 4 μCi/mg (Amersham Pharmacia Biotech, Piscataway, NJ)] in the presence and absence of a 200-fold excess of diethylstilbestrol for 16–18 h at 4 C. Free radioactivity was removed by adding 5% dextran-coated charcoal. In the whole cell binding assay, 5×10^5 MCF-7 cells were plated into six-well dishes in IMEM containing 5% CCS. The medium was subsequently replaced with phenol red-free IMEM containing 5% CCS. After 2 d in estrogen-depleted medium, the cells were incubated for 16–18 h at 4 C with various concentrations of ^{63}Ni and ^{57}Co (10^{-12}–10^{-7} M) in the presence or absence of a 200-fold molar excess of diethylstilbestrol. Free radioactivity was removed by washing the cells. The cells were disrupted by four freeze-thaw cycles, and the amounts of ^{63}Ni and ^{57}Co bound to ERα were measured by scintillation counting. The data were analyzed by the method of Scatchard.

To measure the binding of radioactive nickel, cobalt, or estradiol to ERα mutants, COS-1 cells were plated into six-well dishes and transiently transfected with either wild-type or mutant ERα. The transfected cells were incubated at 4 C for 16 h with various concentrations of ^{63}Ni, ^{57}Co, or [^{3}H]estradiol (10^{-12}–10^{-7} M) in the presence or absence of a 200-fold excess of diethylstilbestrol. Free radioactivity was removed by aspiration, and the cells were disrupted by four freeze-thaw cycles. The amount of bound radioactivity was quantified by scintillation counting, and the data were analyzed as described above.

Results

Effect of metals on the growth of MCF-7 cells

To determine whether metals mimic the effects of estradiol on cell proliferation, the ability of the metals to promote anchorage-dependent growth of MCF-7 cells was determined. Cells were treated with 10^{-9} M estradiol and/or 10^{-6} M metal salt in the presence or absence of 500 nM ICI 182,780, and the number of cells was counted at different times. The results are presented in Fig. 1. Copper, cobalt, nickel, lead,

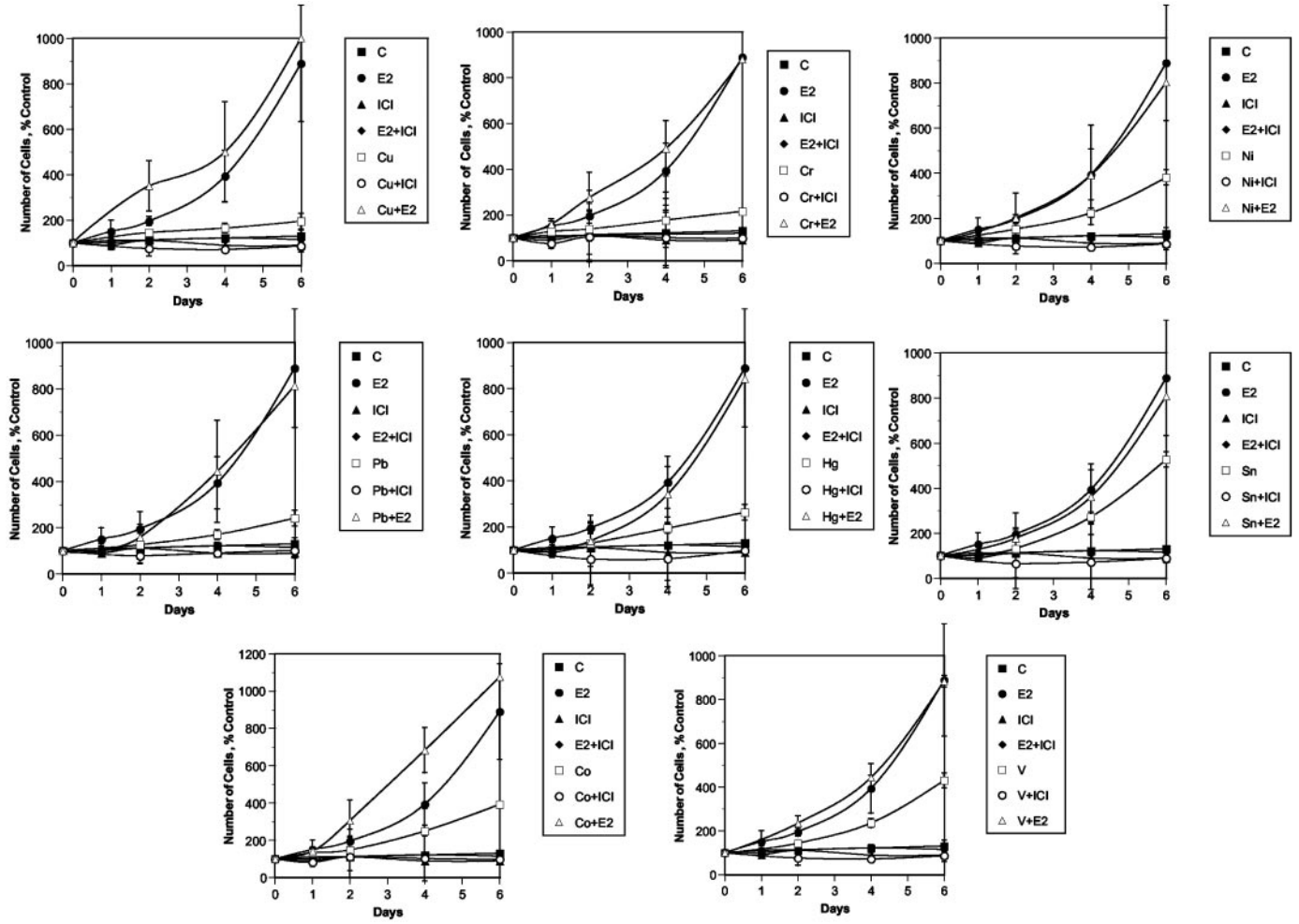

FIG. 1. Effects of metals on the growth of MCF-7 cells. MCF-7 cells were plated and grown in IMEM supplemented with 5% FCS. When the cells reached 40% confluence, the medium was changed to phenol red-free medium supplemented with 5% CCS for 2 d. Cells were treated with 10^{-9} M estradiol and/or 10^{-6} M metal salt in the presence or absence of 5×10^{-7} M ICI 182,780. After treatment, cells were counted at different time points. The results are presented as a percentage of the value in untreated cells and represent the mean ± SD of three different experiments performed in triplicate.

mercury, tin, chromium (II), and vanadate significantly stimulated the growth of MCF-7 cells compared with that of cells grown in estrogen-depleted medium. A 2- to 5-fold increase in cell number was observed after 6 d of treatment with the metals compared with a 9-fold increase in cell growth after estradiol treatment. As expected, the antiestrogen blocked estradiol-stimulated growth. The antiestrogen also blocked metal-stimulated proliferation, suggesting that the growth-promoting effects of the metals are mediated by the ER. When cells were treated with estradiol and copper or cobalt, there was an additive effect on cell growth ($P < 0.05$).

Effect of metal treatment on the concentration of ERα protein

To determine whether the metals demonstrate estrogen-like effects on the expression of ERα, an enzyme immunoassay was employed to measure the concentration of ERα protein. MCF-7 cells were treated for 24 h with 10^{-6} M metals in the presence and absence of 10^{-9} M estradiol (Fig. 2). In agreement with previous observations (13), treatment with estradiol resulted in a 62% decrease in ERα protein (13). Similar to estradiol treatment, the concentration of ERα decreased by approximately 40–60% from 415 fmol/mg protein in control cells to 166–249 fmol/mg protein in cells treatment with copper, cobalt, nickel, lead, mercury, tin, chromium, or vanadate. However, treatment with chromium (III) or zinc (II) did not alter the concentration of ERα protein, suggesting that some, but not all, metals mimic the effects of estradiol. Treatment of cells with estradiol and copper, cobalt, lead, or vanadate had an additive effect on ERα expression ($P < 0.05$). However, no additional effect on ERα was observed when cells were treated with estradiol and nickel, mercury, tin, or chromium (II).

Effect of metals on the steady state amount of ERα mRNA

To determine whether the reduction in ERα protein parallels a reduction in the steady state amount of ERα mRNA, an RNase protection assay was performed. MCF-7 cells were treated with 10^{-6} M metal in the presence and absence of 10^{-9} M estradiol, and the effects of treatment on the steady state amount of total ERα mRNA were measured. In this experiment the amount of ERα mRNA was quantified by phosphorimaging and normalized to the amount of the acidic ribosomal phosphoprotein PO mRNA, and the data are presented in Fig. 2 as a percentage of the control ratio of the ERα signal to the ribosomal protein signal. Treatment with 10^{-9} M estradiol resulted in a 70% decrease in ERα mRNA. Treatment of MCF-7 cells with copper, cobalt, nickel, lead, tin, chromium, or vanadate resulted in a 40–60% decrease in ERα mRNA. The decrease in ERα mRNA correlated with the magnitude of the effect of the metals on the amount of ERα protein. Treatment with a combination of estradiol and copper, cobalt, or lead, but not with nickel, mercury, tin, or vanadate, resulted in an additive effect on ERα mRNA ($P < 0.005$).

Effect of metal treatment on the concentration of progesterone receptor protein and mRNA

To determine whether the metals also mimic the effects of estradiol on the expression of progesterone receptor protein, an enzyme immunoassay was performed. MCF-7 cells were treated with 10^{-6} M metal salt and/or 10^{-9} M estradiol for 24 h, and the concentration of progesterone receptor protein was measured (Fig. 3A). Treatment with estradiol resulted in a 6-fold increase in progesterone receptor over control values. In response to treatment with copper, cobalt nickel, lead, mercury, chromium, or vanadate, the progesterone receptor concentration increased 1.8- to 4-fold compared with control

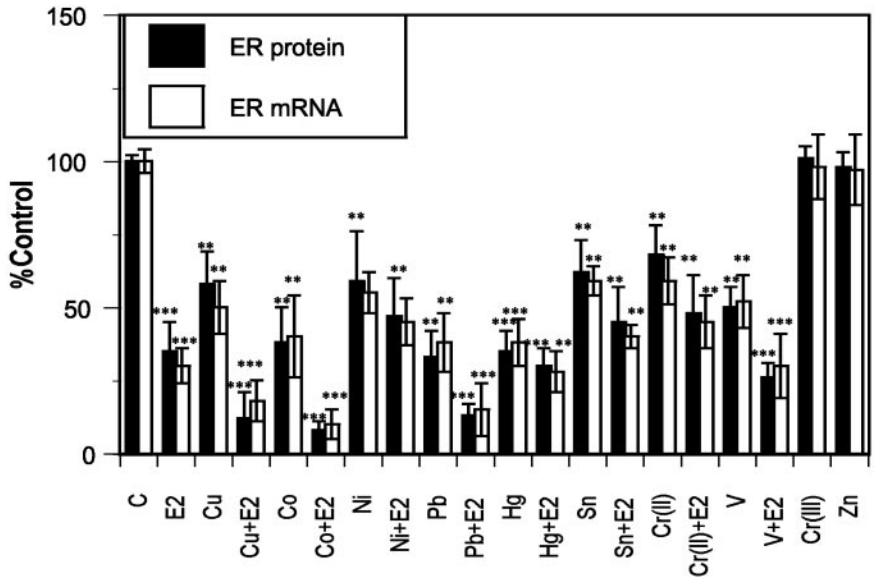

FIG. 2. Effects of metals on ERα protein and mRNA. MCF-7 cells were grown as described in Fig. 1. At 80% confluence, the medium was changed to phenol red-free IMEM and 5% CCS. Cells were grown in this medium for 2 d and then treated for 24 h with 10^{-9} M estradiol and/or 10^{-6} M metal salt in the presence or absence of 5×10^{-7} M ICI 182,780. ERα protein (■) was determined using an enzyme immunoassay and ERα mRNA (□) was determined by an RNase protection assay as described in *Materials and Methods*. Results are expressed as a percentage of the value in untreated cells and represent the mean ± SD of at least three experiments. **, $P = 0.02$; ***, $P = 0.005$.

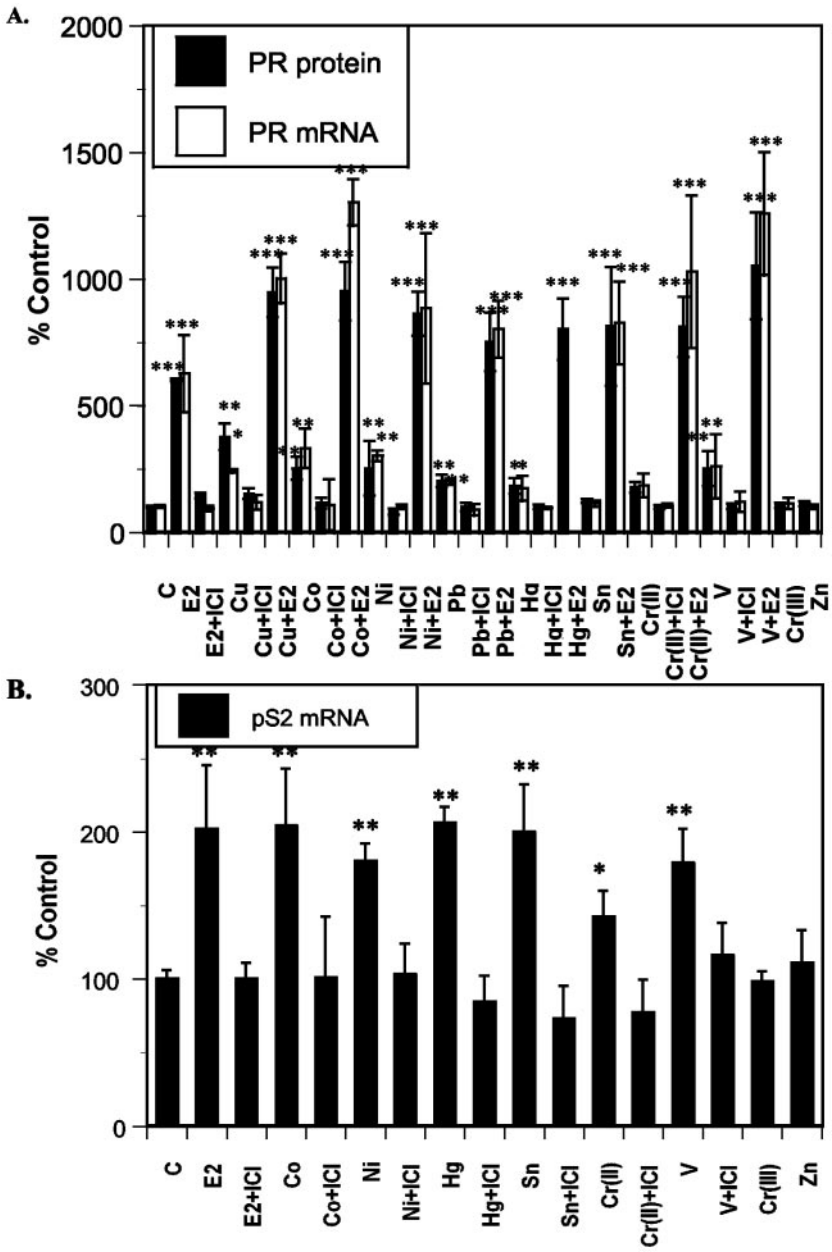

FIG. 3. Effects of metals on ERα activity. A, Effect of metals on progesterone receptor protein and mRNA. B, Effect of metals on pS2 mRNA. MCF-7 cells were grown and treated as described in Fig. 2. The progesterone receptor protein (A, ■) was measured by an enzyme immunoassay, and the steady state amounts of progesterone receptor mRNA (A, □) and pS2 mRNA (B) were determined by an RNase protection assay as described in *Materials and Methods*. The amounts of progesterone receptor and pS2 mRNA data were normalized to the amounts of glyceraldehyde-3-phosphate dehydrogenase mRNA and acidic ribosomal phosphoprotein PO mRNA, respectively. The results were expressed as a percentage of the value in untreated cells and represent the mean ± SD of three experiments. *, $P = 0.05$; **, $P = 0.02$; ***, $P = 0.005$.

values. Only tin did not significantly increase progesterone receptor protein. To determine whether the effects were mediated by ERα, the ability of the antiestrogen ICI-182,780 (5×10^{-7} M) to block the effects of the metals was tested. As expected, the antiestrogen blocked the effect of estradiol. The antiestrogen also blocked the effects of the metals, suggesting that the effects of these compounds are mediated by ERα. Treatment of cells with both estradiol and metal had an additive effect on progesterone receptor induction ($P < 0.05$). To determine whether the metals induced progesterone receptor mRNA, an RNase protection assay was employed. Similar to the results obtained for progesterone receptor protein, the metals increased progesterone receptor mRNA (Fig. 3A). Metal induction of progesterone receptor mRNA was also inhibited by the antiestrogen and was additive with estradiol. Chromium (III) and zinc had no effect on the expression of progesterone receptor.

Effect of metals on the steady state amount of pS2 mRNA

To determine whether metals regulate the expression of other estrogen-responsive genes, MCF-7 cells were treated

with either 10^{-6} M metal salt or 10^{-9} M estradiol for 24 h, and the amount of pS2 mRNA was measured by an RNase protection assay. In this study estradiol induced a 2-fold increase in pS2 mRNA. Similar to estradiol, all of the metals, including tin, induced pS2 mRNA by 1.6- to 2.8-fold over control values (Fig. 3B). As in the case of progesterone receptor, the effects of metals on the expression of pS2 mRNA were blocked by 5×10^{-7} M ICI-182,780, suggesting that the effects of metals are mediated by ERα.

Activation of ERα by metal

To measure the ability of the metals to activate endogenously and exogenously expressed ERα, transient transfection assays were employed. In the first assay, the ability of metals to activate endogenous ERα was tested by transiently transfecting MCF-7 cells with an estrogen response element-luciferase reporter construct (Fig. 4A). In the second assay, the ability of metals to activate exogenously expressed ERα was measured by transiently cotransfecting COS-1 cells with a wild-type ERα expression vector and an estrogen response element-CAT reporter construct (Fig. 4B). The transfected cells were treated with either 10^{-9} M estradiol or 10^{-6} M metal, and reporter activity was measured in the presence or absence of antiestrogen. Treatment with estradiol stimulated luciferase and CAT activities by approximately 7- to 13-fold, respectively, whereas treatment with the metals produced a 1.5- to 3-fold increase in luciferase activity and a 2- to 4-fold increase in CAT activity. The metal-induced increase in reporter activity was blocked by the antiestrogen, demonstrating that the metals activate endogenously as well as an exogenously expressed receptor.

To measure the estrogenic potency of the metals, the 50% effective concentration (EC_{50}) of each metal was determined from dose-response curves performed in MCF-7 cells transiently transfected with the luciferase reporter construct. Transfected cells were treated with concentrations of estradiol or metal from 10^{-12}–10^{-5} M. The EC_{50}, defined as the concentration that produces a 50% increase in luciferase activity, was determined for each metal (Table 1). The relative potency of the metals was then determined as the ratio of the EC_{50} of estradiol to the EC_{50} of the metal. The EC_{50} values for the metals ranged from 2×10^{-9} M for nickel to 1×10^{-8} M for copper compared with the EC_{50} value for estradiol of 2×10^{-9} M with the relative potencies of the metals varying from 0.2–1.0.

TABLE 1. Estrogenic potency of metals

Compounds	EC_{50} (M)[a]	Relative potency[b]
Estradiol	2×10^{-9}	1
Copper	1×10^{-8}	0.2
Cobalt	7×10^{-9}	0.29
Nickel	2×10^{-9}	1
Lead	7.5×10^{-9}	0.27
Mercury	7.5×10^{-9}	0.27
Tin	7.3×10^{-9}	0.28
Chromium	7.5×10^{-9}	0.27
Vanadate	8×10^{-9}	0.25

[a] EC_{50} is defined as the concentration that induces a 50% increase in luciferase activity.

[b] The relative potency was determined as the ratio of the EC_{50} of estradiol to the EC_{50} for each metal.

Interaction of metals with the hormone-binding domain of ERα

To identify the region of ERα involved in activation by the metals, a chimeric receptor containing the hormone-binding domain of ERα was employed. The chimeric receptor, GAL-ER, contains the DNA-binding domain of the yeast transcription factor GAL4 fused to the hormone-binding domain of ERα. Stimulation of transcription by GAL-ER chimera from a GAL4-responsive CAT reporter gene is dependent upon estradiol (22). When the chimeric receptor GAL-ER and the GAL4-CAT reporter construct were transiently cotransfected into COS-1 cells, and the cells were treated with estradiol, there was a 4.4-fold increase in CAT activity (Fig. 4B). Treatment with 10^{-6} M metal resulted in a 2- to 5.8-fold increase in CAT activity, suggesting that the metals activate ERα through the hormone-binding domain. To determine whether activation by metals requires a functional AF-2 domain, an ERα mutant that has an intact AF-1 domain, but is mutated in the AF-2 domain, was used. The ERα mutant, referred to as ERTAF-1, is mutated at residues 538, 542, and 545 (24). A second mutant containing the AF-2 mutations and also lacking the AF-1 domain (ER-null) was used as a control. The AF-2 mutants were transiently transfected with an estrogen response element-CAT reporter gene into COS-1 cells, and CAT activity was determined after treatment with estradiol or metal. Similar to estradiol, treatment of the AF-1 mutants with 10^{-6} M metal resulted in no increase in CAT activity (data not shown), suggesting that a functional AF-2 domain was required for activation of ERα by the metals. To establish whether the interaction of ERα with metals altered its interaction with coactivators, MCF-7 and CHO cells were transiently cotransfected with a splice variant of AIB1 that lacks exon 3 (AIB1 δ3). This isoform of AIB1 is more effective than full-length AIB1 in promoting the transcriptional activity of ERα in both MCF-7 and CHO cells (25). As expected, the presence of the coactivator enhanced the transcriptional activity of ERα in MCF-7 cells upon treatment with estradiol. There was a 6-fold increase in luciferase activity in the absence of AIB1 δ3 and an 18-fold increase in the presence of the coactivator (Fig. 4A). However, AIB1 δ3 did not enhance the transcriptional activity of ERα upon treatment with metals. In the presence of AIB1 δ3, metal treatment did not produce a significant increase in luciferase activity compared with treatment with metals in the absence of the coactivator. In contrast to the results observed in MCF-7 cells, the coactivator produced a significant enhancement of metal-induced activation of ERα in CHO cells. When CHO cells were transiently cotransfected with ERα and an estrogen-responsive luciferase reporter gene in the presence and absence of AIB1 δ3, the coactivator produced a 2- to 4-fold enhancement of the metal response, suggesting that metals do not interfere with the interaction of ERα and coactivators.

Activation of ERα mutants by metals

Metals are capable of forming high affinity complexes directly or indirectly with many different amino acids, including cysteines. The hormone-binding domain of ERα contains four cysteines at positions C381, C417, C447, and C530. To test the roles of these cysteines in the interaction with the

metals, each cysteine was mutated to alanine (16). The cysteine mutants C381A, C417A, C447A, and C530A were transiently cotransfected with an estrogen-responsive CAT construct into COS-1 cells, and the cells were treated with 10^{-9} M estradiol or 10^{-6} M metal. The amount of CAT activity was measured, expressed as the percent conversion, and normalized to the amount of β-galactosidase activity (Fig. 4D). After treatment with heavy metals, there was an approximately 2- to 4-fold increase in CAT activity with mutants C417A and C530A. In contrast to the effects observed with these mutants, the metals failed to activate the mutants C381A and C447A, suggesting that cysteines C381 and C447 may be involved in activation of ERα by the metals. To demonstrate that the mutation of cysteine to alanine did not interfere with the activity of the receptor, the transiently transfected cells were treated with 10^{-9} M estradiol. After hormone treatment, there was an approximately 10- to 17-fold increase in CAT activity with all mutants. These results corroborate previous studies employing these mutants that demonstrate that mutation of cysteines in the hormone-binding domain to alanine does not alter the ability of estradiol to *trans*-activate the receptor (16). In addition to cysteines, metals are capable of interacting with histidines. Histidine H524 has previously been shown to interact with estradiol (26) as well as cadmium, arsenite, and selenite (4–6). Mutation of histidine H524 to alanine resulted in the complete loss of activation of ERα by the metals, but did not alter the ability of estradiol to *trans*-activate the receptor (Fig. 4E). To identify other possible interaction sites within the hormone-binding domain of ERα, aspartic acid D538 and glutamic acid E523 were mutated to asparagine and glutamine, respectively. With the exception of vanadate, the metals failed to activate the receptor, suggesting that aspartic acid D538 and glutamic acid E523 interact with the divalent metals. Vanadate activated E523Q and D538N, resulting in an approximately 4-fold increase in CAT activity. As vanadate has a negative charge, it would be expected to interact with positively charged amino acids rather than negatively charged amino acids. Possible candidate amino acids include lysines K529 and K531 and asparagine N532 that were mutated to glutamic acid and aspartic acid, respectively (17–19). Vanadate did not activate mutants K529Q K531Q N532D, K529Q K531Q, or N532D (Fig. 4F), suggesting that asparagine N532 and at least one of the lysines, K529 and/or K531, may also play a role in the interaction of vanadate with ERα. With the exception of H524, estradiol treatment of all mutants resulted in an approximately 10- to 17-fold increase in CAT activity, suggesting that mutation of these amino acids did not interfere with the activity of ERα. Estradiol treatment of H524A resulted in 7-fold induction of CAT activity, in agreement with previously published results (18).

Effect of metals on the binding of estradiol to ERα

To determine whether metals blocked estradiol binding to ERα, the effects of these compounds on hormone binding were measured using a single dose ligand binding assay. Human recombinant ERα was treated with 10^{-6} M metal salt for 1 h at 4 C. The ability of ERα to bind hormone was then assayed by incubating the extract with 10 nM [^{3}H]estradiol in the presence or absence of a 200-fold molar excess of diethylstilbestrol for 2 h at 37 C. As shown in Fig. 5, all of the heavy metals tested, with the exception of Cr (III) and Zn (II), blocked the binding of estradiol to the receptor. To further characterize the binding of the metals, a multiple dose ligand binding assay was performed. Cytosolic extracts from MCF-7 cells were treated with 10^{-6} M metal for 1 h at 4 C. Various concentrations of [^{3}H]estradiol (10^{-12}–10^{-7} M) were added in the presence or absence of a 200-fold molar excess of diethylstilbestrol. The apparent binding affinity and binding capacity of the ER were determined according to the method of Scatchard (23) (Table 2). In the absence of metal, estradiol bound to the receptor with an equilibrium dissociation constant of $3.06 \pm 2.88 \times 10^{-10}$ M (n = 3). In the presence of metal, the dissociation constant of estradiol was unchanged, but the number of binding sites decreased. These results suggest that the metals compete with estradiol for binding to ERα in a noncompetitive manner.

Metal binding to ERα and ERα mutants

To characterize the binding of the metals to ERα, Scatchard analysis was performed using radioactive cobalt and nickel and human recombinant ERα. Recombinant ERα was incubated with various concentrations of ^{57}Co or ^{63}Ni (10^{-12}–10^{-7} M) in the presence and absence of a 200-fold excess of diethylstilbestrol, and the results are shown in Table 3. The metals bound to ERα with an affinity similar to that of estradiol for the receptor. The dissociation constants for cobalt and nickel were 3×10^{-9} and 2×10^{-9} M, respectively. The binding of cobalt and nickel to ERα in MCF-7 cells was also measured for comparison with the binding to recombinant receptor. Similar to recombinant receptor, the dissociation constants in whole cell extracts for cobalt and nickel were 9.5×10^{-9} and 7×10^{-9} M, respectively. These results demonstrate that there was no significant difference in the binding affinity of cobalt, nickel, or estradiol to ERα.

To determine whether the ERα mutants were capable of binding cobalt and nickel, the mutants were expressed in COS-1 cells, and the specific binding of ^{57}Co and ^{63}Ni was determined using a whole cell binding assay (Table 3). All of the mutants were capable of binding estradiol with high affinity, but at a lower affinity than the wild-type receptor. Radioactive cobalt, on the other hand, bound with high affinity to the ERα mutants C447A, E523A, D538N, and N532D, but not to the C381A and H524A mutants. Radioactive nickel bound only to the N523D mutant. There was no detectable binding of nickel to the C381A, C447A, E523A, H524A, and

TABLE 2. Effect of metals on estradiol binding to ERα

Compound	K_d ($\times 10^{-10}$ M)	B_{max} (% of control)
Control	3.06 ± 2.88	100
$CuCl_2$	3.03 ± 0.37	10 ± 2
$CoCl_2$	3.67 ± 1.52	59 ± 13
$NiCl_2$	4.42 ± 1.52	42 ± 15
Na_3VO_4	1.14 ± 0.92	28 ± 6
$PbCl_2$	1.07 ± 0.81	33 ± 12
$HgCl_2$	3.96 ± 0.72	24 ± 7
$SnCl_2$	3.48 ± 2.02	75 ± 19
$CrCl_2$	2.61 ± 0.88	65 ± 14

B_{max}, Binding capacity.

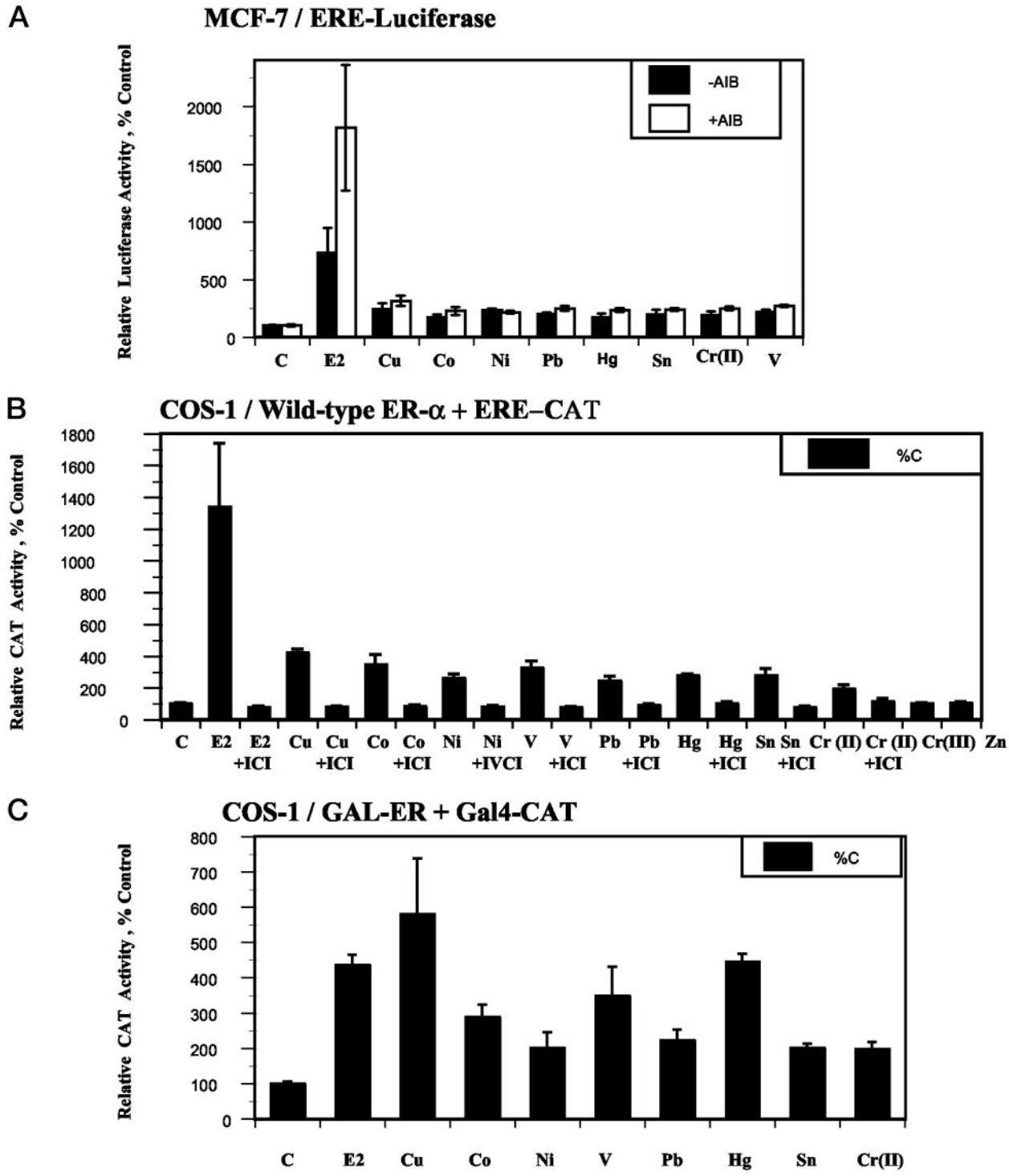

FIG. 4. The ability of metals to activate ERα and GAL-ER. A, Effects of metals on the activity of ERα in MCF-7 cells. B, Effects of metals on the activity of wild-type ERα in COS-1 cells. C, Effects of metals on the activity of the chimeric receptor GAL-ER in COS-1 cells. D, Effects of metals on the activity of ERα mutants C381A, C417A, C447A, and C530A in COS-1 cells. E, Effects of metals on the activity of ERα mutants H524A, D538N, and E523Q in COS-1 cells. F, Effects of metals on the activity of ERα mutants N532D, K529Q.K531Q, K529Q, and K531Q.N532D in COS-1 cells. MCF-7 cells were transiently transfected with an estrogen response element-luciferase reporter gene (A). Wild-type and mutants of ERα were transiently cotransfected with an estrogen response element-CAT or a GAL-4-CAT reporter construct into COS-1 cells (B–F). The transfected cells were treated for 24 h with either 10^{-9} M estradiol or 10^{-6} M metal salt in the presence or absence of 5×10^{-7} M ICI 182,780. Luciferase and CAT activities were measured as described in *Materials and Methods*. The results were normalized to the *Renilla* or β-galactosidase activity and expressed as the percentage of reporter gene activity in untreated cells (mean ± SD; n = 3). *, $P = 0.05$; **, $P = 0.02$; ***, $P = 0.005$.

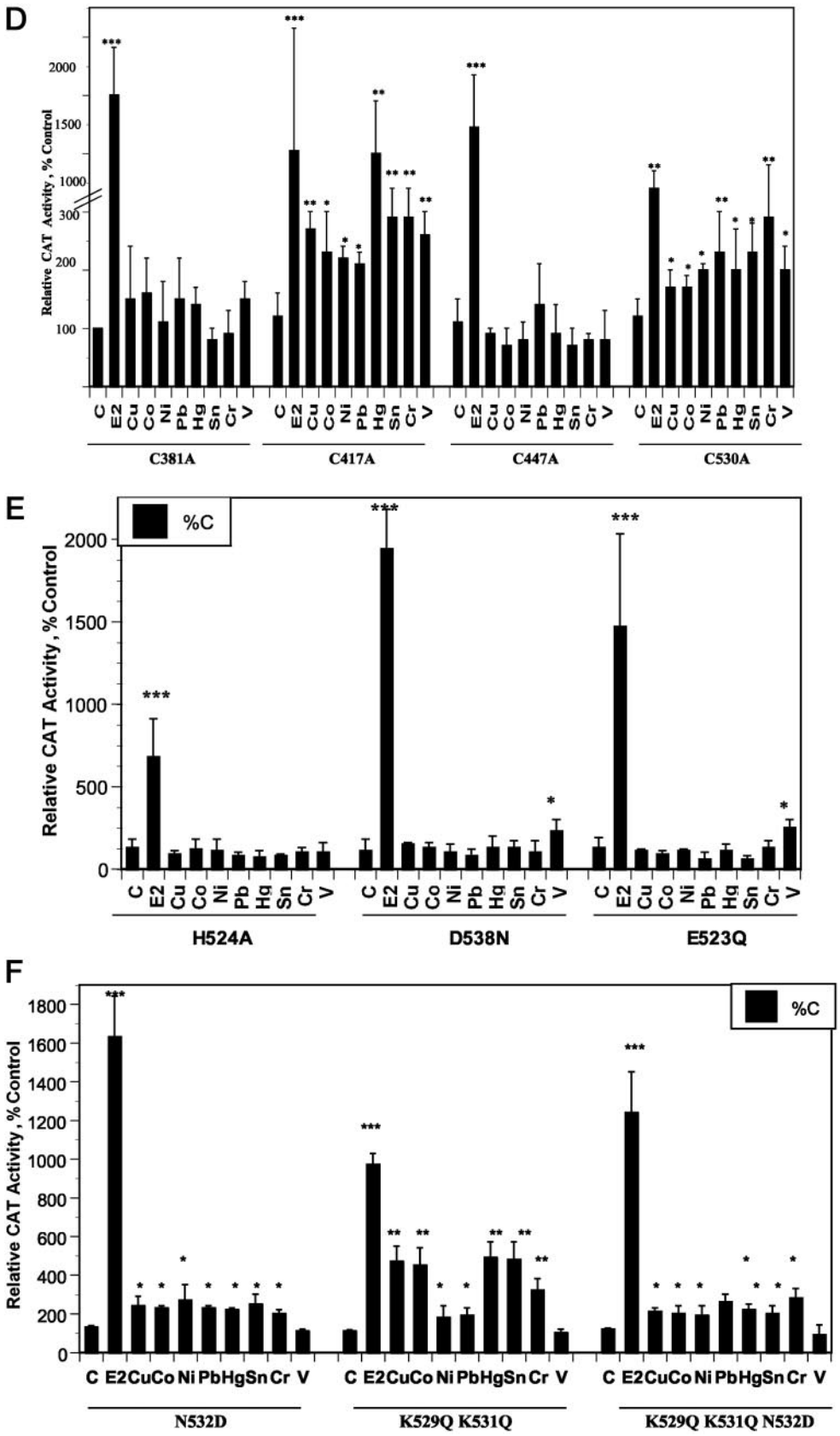

FIG. 4. *Continued.*

TABLE 3. Metal binding to ERα and ERα mutants

ERα	Binding ($\times 10^{-9}$ M)			Literature[a] $[^3H]E_2 \times 10^{-9}$ M
	^{63}Ni	^{57}Co	$[^3H]E_2$	
Recombinant ERα	3 ± 0.9	2 ± 0.8	0.6 ± 0.1	NT
Wild type	9.5 ± 3	7 ± 1	NT	0.24 ± 0.5
C381A	0	0	60 ± 21	80
C447A	0	8 ± 0.9	66 ± 17	84
E523A	0	8 ± 2	25 ± 16	50
H524A	0	0	40 ± 9	140 ± 4
D538N	0	7 ± 1.8	54 ± 16	37
N532D	8 ± 2.4	8.5 ± 1.6	NT	NT

E_2, Estradiol; NT, not tested.
[a] Refs. 48 and 49.

D538N mutants. The inability of some of these mutants to bind metals suggests that these amino acids play a role in the binding as well as the activation of ERα.

Discussion

We have previously shown that the metal cadmium as well as arsenite and selenite mimic the biological functions of estradiol in breast cancer cells by activating the ER (3–5). The purpose of this study was to determine whether other metals mimic the effects of estrogens and to explore the mechanism(s) underlying their estrogen-like function. The present study demonstrates that the divalent metal chlorides of copper, cobalt, nickel, lead, mercury, tin, and chromium as well as the metal anion vanadate have a significant effect on ERα expression and activity. Similar to estradiol, these metals induce the growth of MCF-7 cells, decrease the steady state amounts of ERα protein and mRNA, induce the two estrogen-regulated genes, progesterone receptor and pS2, and activate ERα in transient transfection experiments. The ability of the metals to mimic the functions of estradiol is blocked by an antiestrogen, suggesting that their effects are mediated by ERα. The metals also activate a chimeric receptor containing the DNA-binding domain of the yeast transcription factor GAL4 and the hormone-binding domain of ERα, suggesting that the metals activate ERα through the hormone-binding domain. Moreover, studies using AF-2 mutants demonstrate that activation of ERα by the metals requires a functional AF-2 domain. Additional evidence that the hormone-binding domain is the target site is demonstrated by the ability of the metals to inhibit the binding of estradiol to the receptor. Mutational analysis and binding assays identified several amino acids as potential interaction sites, suggesting that the metals activate ERα through the formation of a high affinity complex within the hormone-binding domain of the receptor.

In proteins, metals serve several functions, including participation in catalytic reactions and stabilization of protein structure. Through their interactions with different amino acids, metals may promote local folding, as in the case of the zinc fingers of the ER, or assembly of different regions of the protein into one domain (27). Similar to other steroid receptors, the hormone-binding domain of ERα contains 12 α-helixes (H1-H12) folded into a 3-layered antiparallel α-helical sandwich (26, 28–32). The central core layer contains 3 α-helixes composed of H1–4, H7, H8, and H11, and is flanked by helix H12. Upon binding of the ligand, several structural

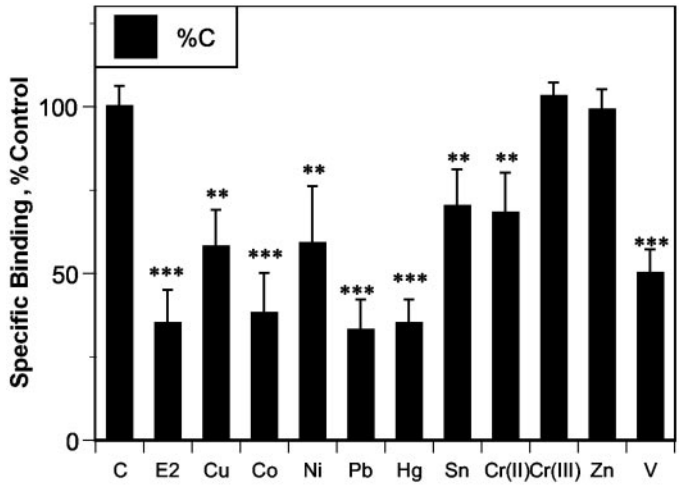

FIG. 5. Effects of metals on estradiol binding to ERα. Human recombinant ERα (4×10^{-9} M) was preincubated for 1 h at 4 C with 10^{-6} M metal salt and then treated with 10^{-8} M $[^3H]$estradiol in the presence or absence of a 200-fold molar excess of diethylstilbestrol for 2 h at 37 C. The amount of specific binding was determined as described in *Materials and Methods* and expressed as a percentage of the value in untreated cells. Results represent the mean ± SD of three experiments. *, $P = 0.05$; **, $P = 0.02$; ***, $P = 0.005$.

changes occur, resulting in the rearrangement of helixes H3, H6, H11, and H12. The most striking conformational change is the repositioning of H12 over the central core and the formation of a salt bridge between H4 and H12. The positioning of helix H12 over the central core generates a binding site for coactivators (33). The divalent metal ions copper, cobalt, nickel, lead, mercury, tin, and chromium, but not the divalent zinc ion or the trivalent chromium ion, appear to activate ERα through a mechanism involving cysteines C381 and C447, histidine H524, and the negatively charged amino acids glutamic acid E523 and aspartic acid D538. Vanadate also interacts with cysteines C381, C447, and histidine H524, but, consistent with its negative charge, interacts with the positively charged amino acids asparagine N532 and lysines K529 and/or K531 instead of the negatively charged amino acids. These target amino acids are located on helixes H4, H8, and H11 and in the loop between H11 and H12. Cysteines C381 and C447 are located on helixes H4 and H8, respectively. Glutamic acid E523 and histidine H524 are located on helix H11 and are in close proximity to estradiol when the hormone is bound to the receptor. Asparagine N532, aspartic

acid D538, and lysines K529 and K531 are located in the loop between H11 and H12. It is not certain whether these amino acids form the metal-binding site, possibly through a direct interaction with the metal or an indirect interaction with water, or whether some of these amino acids recruit the metal to the binding site, *i.e.* the metal may bind to ERα in a 2-step mechanism. In the first step of the latter model, the metal would bind to amino acids on helix H11 and in the loop between helixes H11 and H12. Metal binding would reposition helix H12 with respect to H11, resulting in the dissociation of proteins, such as heat shock proteins, from the receptor. In the second step, the metal would bind to C381 and C447 and possibly other amino acids and stabilize the active form of the receptor. Alternatively, in the first model, two molecules of the metal would bind to ERα. One molecule would bind to C381 and C447, and the second molecule would bind to H11 and the adjacent loop, resulting in a conformational change similar to that observed upon hormone binding. However, previous studies with cadmium support a 2-step model. Similar to the divalent metals tested in this study, cadmium interacts with cysteines C381 and C447, histidine H524, glutamic acid E523, and aspartic acid D538, but binds to ERα in a one to one stoichometry, suggesting a 2-step model of metal binding to the receptor. This model remains to be tested.

The ability of metals to bind with high affinity and activate ERα suggests that at environmentally relevant doses these compounds may pose a risk for endocrine-related diseases. In fact, exposure to metals is associated with endocrine imbalances and significant reproductive toxicity (34, 35). In women, exposure to lead and mercury is linked to infertility, miscarriage, preeclampsia, pregnancy hypertension, premature delivery, and an increase in menstrual cycle disturbances. In experimental animals, exposure to these metals results in an inhibition of menstruation, ovulation, and follicular growth; a delay in vaginal opening; and an decrease in pregnancy (36, 37). Copper is also associated with the actions of estrogens. Copper accumulates in normal and neoplastic estrogen target tissues, such as uterus and mammary gland (38, 39), and appears to modulate the sensitivity of these tissues to both estrogens and antiestrogens. In women the increase in estrogen during pregnancy is linked to increased levels of circulating copper. In rats, copper deficiency leads to infertility, and in rabbits the injection of copper induces ovulation; an effect that is enhanced by pretreatment with estrogens (40). In addition to endocrine-related diseases, women working in dentistry also have an increased frequency of precancerous lesions of the cervix that correlates with the length of employment (36). In animal studies, nickel, cobalt, mercury, lead, and chromium (VI), but not chromium (III), induce carcinomas in the breast as well as in the kidneys, lungs, liver, and pancreas (41–45). Vanadate, on the other hand, blocks the induction of mammary tumors in chemically treated mice (36).

Environmental exposure to metals is significant. In water and soil, the concentrations of chromium, mercury, and copper are 1–800 μg/liter and 40–459 mg/kg, respectively (46–48). In fish, the amounts of nickel, chromium, mercury, lead, and copper range from 81–328 mg/g, and in grain, the amount of copper ranges from 1–14 μg/g (49). In humans, exposure to metals occurs primarily through dietary sources of food and water (50, 51), air, cigarette smoke (52), and occupational exposure (50, 52, 53) and can lead to significant accumulation in the body. The average daily intake of chromium, mercury, and nickel is estimated to range from 0.28–25 μg/d, whereas the daily intake of copper ranges from 1.46–1.63 mg/d (54). Lead exposure results in significant accumulation in hair and toenails (3.8–10.1 μg/g) (55) and in breast milk (36 mg/liter) (49). The concentrations of copper, cobalt, vanadate, and tin appear to be significantly elevated in the serum of breast cancer patients and vary with the stage of the disease; the highest levels are observed in advanced stages (56–62). Serum levels of copper are also higher in premenopausal than in postmenopausal breast cancer patients. A marginally significant association is observed between toenail levels of chromium and breast cancer risk in postmenopausal women, but an inverse association is found among premenopausal women (63). Although the precise roles of metals and metalloids in breast cancer and other endocrine-related diseases remain to be determined, their ability to function as potent estrogens suggests that they may be an important class of endocrine disrupters. Because exposure to metals is widespread, the elucidation of their roles in the etiology and development of hormone-related diseases such as breast cancer may have significant implications in risk reduction and disease prevention.

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