Effects of Tobacco Smoke Condensate on Estrogen Receptor-α Gene Expression and Activity

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Metallo-estrogens are a new class of potent environmental estrogens. This study investigates whether tobacco smoke condensate (TSC), which contains metals and metalloids, elicits estrogen-like effects at environmentally relevant doses. Treatment of human breast cancer cells, MCF-7, with 40 μg/ml TSC resulted in a 2.5-fold stimulation of cell growth. TSC decreased the concentration of estrogen receptor (ER)-α protein and mRNA (63 and 62%, respectively), and increased the expression of the estrogen-regulated genes, progesterone receptor and pS2 (5- and 2-fold, respectively). In addition, TSC activated the estrogen receptor (ER) and increased the expression of progesterone receptor and complement C3 in the uterus (2- and 26-fold) and mammary gland (4.4- and 15-fold). Both the in vitro and in vivo TSC effects were blocked by the antiestrogen ICI 182,780, suggesting the involvement of ER. Collectively, these results provide strong evidence that low doses of TSC, acting through the hormone binding domain, exert estrogen-like effects in cell culture and animals. (Endocrinology 148: 4676–4686, 2007)

Breast cancer is one of the most frequently diagnosed cancers, with a lifetime risk of one in eight women in the more developed countries (1). Neither changes in established risk factors nor screening practices can account for the persistent 1% annual increase in the incidence of breast cancer, nor can they explain geographic variations in the prevalence of the disease (2–5). The development of breast cancer is closely related to endogenous exposures to circulating estrogens, suggesting that molecules that bind to and activate the estrogen receptor (ER)-α can potentially increase the risk for the disease. We have identified a new class of potent environmental estrogens, referred to as metallo-estrogens, that include the bivalent metal cations cadmium, cobalt, copper, nickel, chromium, lead, mercury, and tin, and the metal/metalloid anions arsenite, selenite, and vanadate (6–12). These metals and metalloids mimic the effects of estradiol by activating ER-α through a high-affinity interaction with the hormone binding domain of the receptor (7–9, 11). The ability of these metals and metalloids to activate the ER suggests that environmental exposure to metallo-estrogens may increase the risk of breast cancer.

In the general population, smoking is an important route of exposure to heavy metals and metalloids, suggesting that cigarette smoking may lead to an increased risk of breast cancer, in part, due to the estrogen-like activity of the metal components. In fact, there is conflicting, but suggestive, epidemiological evidence that active and passive cigarette smoking is linked to an increased risk of the disease. Although tobacco smoke contains several metals and metalloids, including arsenic, cadmium, chromium, copper, cobalt, lead, mercury, nickel, tin, and vanadium (reviewed in Ref. 13), it is a complex mixture of both inorganic and organic compounds (14). Because many of the organic chemicals in tobacco smoke are carcinogens (15), they are lipophilic and concentrate in breast tissue, and are activated by enzymes that are expressed in breast epithelial cells, it has been suggested that exposure to the chemical carcinogens in cigarette smoke is an underlying cause of breast cancer. However, the association between chemical carcinogens and breast cancer is not clear (16, 17). The metal components in tobacco are also carcinogens (reviewed in Ref. 18). Arsenic, cadmium, chromium, and copper are group I carcinogens; nickel is a suspected carcinogen, and copper, vanadium, mercury, and lead are probable or possible carcinogens or cocarcinogens. Oxidative stress is thought to be the principal mechanisms by which many, but not all, of these metals initiate tumorigenesis. The ability of these metals and metalloids to also activate ER-α suggests that cigarette smoking may be a risk factor for breast cancer due, in part, to the estrogen-like activity of the metal contaminants. To determine whether cigarette smoke has potent endocrine disrupting activity, the ability of tobacco smoke condensate (TSC) to activate ER-α in breast cancer cells and ovariectomized animals was tested.
Materials and Methods

Tissue culture

Wild-type MCF-7 human breast cancer cells were grown in improved MEM (IMEM) (Life Technologies, Inc./BRL, Rockville, MD) supplemented with 5% fetal calf serum (FCS) (Quality Biological Inc., Gaithersburg, MD). At 80% confluence, the medium was changed to phenol red-free IMEM supplemented with 5% charcoal-stripped calf serum (CCS) (Equitech-Bio, Inc., Kerrville, TX). Cells were maintained in this medium for 2 d before treatment. Cells were treated with either TSC (Murry Pharmaceuticals Inc., Lexington, KY) or estradiol (Sigma Chemicals, St. Louis, MO) in the presence or absence of the steroid antiestrogen CI 182,780 (N-n-butyl-N-methyl-13,17β-dihydroxyoestra-1,3,5(10)-trien-7a-y-lundecamide; Toecris, Baldwin, MO).

TSC

TSC was prepared according to the standardized Federal Trade Commission procedure (35-ml puff volume, 2-sec duration, 1 puff/min, smoked to a butt length within 3 mm of the edge of the tipping paper, with the ventilation holes of the cigarettes left unblocked) using a 20-20-port Philip Morris Automated Smoking machine (Richmond, VA) and standard research cigarettes (KY 2R1) obtained from the University of Kentucky Tobacco Health Research Center to maintain consistency and minimize variables in the cigarettes (TSC lot nos. 1, 2, and 4). To verify the reproducibility of the TSC preparations, we also used a batch of TSC from the Lovelace Respiratory Research Institute in Albuquerque, NM (TSC lot no. 3). The cigarettes for this TSC batch were also obtained from the Tobacco Health Research Center in Lexington, KY, and an AMESA type 1300 automated smoking machine (AMESA, Geneva, Switzerland) was used as the smoke generator. The smoke residues were collected on a Cambridge-type filter (Cambridge Filter Corp., Gilbert, AZ), and, subsequently, the filter pads were extracted with dimethylsulfoxide (DMSO). The smoke condensate contains total particulate matter with a yield of 4%. Although the Federal Trade Commission's parameters simulate the manner in which people smoke cigarettes (19), recent smoking studies show that smokers of low-nicotine cigarettes take between two and four puffs per minute with volumes up to 55 ml to satisfy their demands for nicotine (20).

Anchorage-dependent growth assays

MCF-7 cells were plated at 10^4 cells per well in 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 estrogen-free medium, cells were treated with either 10^-7 m estradiol or 40 m gum/ml TSC. The presence or absence of 5 x 10^-7 m triamcinolone acetonide (TIA) was also evaluated. ER binding assays

Cells were grown as described previously. After 24-h treatment, cells were washed twice with PBS (Life Technologies, Inc./BRL), scraped, and pelleted by centrifugation. Cell pellets were sonicated in a high-salt buffer (10 mm Tris, 1.5 mm EDTA, 5 mm Na2MoO4, 0.4 m KCl, and 1 mm monoothioglycerol with 2 mm leupeptin) (21). 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 or PR protein using specific enzyme immunoassay kits from Abbott Laboratories (North Chicago, IL). The released fragment was purified and used to replace the corresponding fragment of pcDNA3-AIB1, thereby creating pcDNA3-AIB1-3. The major difference between AIB1 and AIB1-3 vectors is only the loss of exon 3. The smaller RT-PCR products generated from MCF-7 cell total RNA were subsequently cloned into pCRII (Invitrogen Corp., Carlsbad, CA) with the use of the flanking KpnI and XhoI sites, thereby creating the expression vector pcDNA3-AIB1. The resulting plasmid was digested with Bam HI and Hpal, recognition sequences that flank the splice sites of AIB1-3 cDNA, and the released fragment was purified and used to replace the corresponding fragment of pcDNA3-AIB1, thereby creating pcDNA3-AIB1-3. ER binding assays

The ability of TSC to block estradiol binding to ERα was determined in cell extracts from MCF-7 cells that were 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 monoothioglycerol, and 2 mm leupeptin. The homogenate was incubated on ice for 30 min and centrifuged at 100,000 x g for 1 h at 4 C (32). The protein concentration of the cell extract was determined by the Bradford method using the Bio-Rad assay dye (Bio-Rad Laboratories Inc., Hercules, CA). Cell extracts were preincubated for 1 h with 40 m gum/ml TSC [2,4,6,7H]Estradiol (10^-12 to 10^-7 m) (84 Ci/mmol, 1 mCi) (Amersham Pharmacia Biotech, Piscataway, NJ) was then added in the presence and

Measurement of ERα, PR, and pS2 mRNA amounts

Total cellular RNA was extracted from cells as described previously (21). The amounts of ERα, PR, and pS2 mRNA were determined by an RNase protection assay. 32P-Labeled antisense RNA (cRNA) was synthesized from a full-length cDNA clone of the human estrogen receptor a (ERα) (22, 23) (accl, a derivative of the O-estradiol PO (36b4) (24), pS2 (22), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (23) using 17 polymerase and from pFP2R50 (PR) using SP6 polymerase (21). Sixty micrograms of total RNA were hybridized for 16 h to the 32P-labeled cRNA and digested with RNase A. The protected cRNA probes were resolved on 6% polyacrylamide gels. The bands were visualized by autoradiography and quantified by phosphor imaging using a Molecular Imager 445 SI Molecular Dynamics; Applied Biosystems, Foster City, CA). The amounts of ERα and pS2 mRNA were normalized using 36B4 as an internal control. The amounts of PR mRNA were normalized to GAPDH.
abundance of a 200-fold m excess of diethylstilbestrol (DES) (Sigma) 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. Data were analyzed by the method of Scatchard (33).

The ability of TSC to block estradiol binding was also tested using recombinant human ER-α (FamVera Corp., Madison, WI). Recombinant ER-α (4 × 10−8 M) was preincubated for 1 h on ice with several concentrations of TSC. [3H]Estradiol (10−8 M) was then added in the presence and absence of a 200-fold m excess of DES 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.

Animal studies

For in vivo experiments, female Sprague Dawley rats (Harlan, Indianapolis, IN) were used. Animals were housed under a 12-h light, 12-h dark cycle. Animal care was in accordance with a Georgetown University Institutional Animal Care and Use Committee approved protocol. Animals were ovariectomized by the vendor at 28 d of age and allowed to recover for 2 wk before treatment with TSC, estradiol, or the antiestrogen ICI 182,780. TSC, dissolved in DMSO, was administered ip. One group of animals received a single dose of TSC (10 mg/kg bw); the second group of animals received the same dose of TSC (10 mg/kg bw), administered on 2 consecutive days. An estradiol pellet (60 μg/kg, 30-d release; Innovative Research of America, Sarasota, FL) was implanted sc. The antiestrogen, ICI 182,780 (Tocris), was dissolved in peanut oil and given ip at a dose of 500 μg/kg. Animals were euthanized 5 d later, and uteri and mammary glands were dissected out for RNA extraction. The effects of TSC on uterine wet weight, PR mRNA, and complement C3 mRNA in uteri and mammary gland were examined.

Real-time RT-PCR assay

Total RNA was extracted from tissues with RNA STAT-60 (Tel-Test, Friendswood, TX). Pellets were suspended in 0.5 ml water, and 4 μl was used as substrate in the Platinum qRT-PCR Thermoscript One Step System (Invitrogen Corp.). Besides the supplied reaction buffer, the reaction mixture included a 300 nm final concentration of specific gene primer and GAPDH primer, a 200 nm final concentration of the specific gene probe labeled with FAM fluorescent dye, a 200 nm final concentration of the GAPDH probe labeled with Cy5 fluorescent dye, and 1 μl platinum Taq polymerase/Thermoscript reverse transcriptase mix. The RT-PCR conditions were: 45 min at 54 C, followed by 5 min at 95 C and 50 cycles of 30 sec at 95 C, 30 sec at 54 C, and 30 sec at 68 C. Fluorescent data were collected during the 68 C step using the iCycler iQ Detection System (Bio-Rad Laboratories, Inc.). Data were collected during the 68 C step using the iCycler iQ Detection System (Bio-Rad Laboratories, Inc.). The nucleotide sequences of the primers and probes are shown below (Table 1). As standards we used a series of two-tailed unpaired Student’s t tests with equal variances to determine the statistical significance or differences in cell number, ER, and PR protein or mRNA, pS2 mRNA, C3 mRNA, or reporter gene activity among treatment groups compared with control. A P value < 0.05 was deemed significant (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

Results

Effect of TSC on the growth of MCF-7 cells

To ask whether TSC mimics the effect of estradiol on cell growth, the effect on the anchorage-dependent growth of MCF-7 cells was determined. Cells were treated with 10−9 m estradiol or 40 μg/ml TSC, in the presence or absence of 5 × 10−7 m the antiestrogen ICI 182,780 and the number of cells was counted at different times. The results are presented in Fig. 1. TSC stimulated the growth of MCF-7 cells when compared with cells grown in estrogen-depleted medium. A 2.5-fold increase (P < 0.05) in cell number was observed after 6-d treatment compared with a 8.9-fold increase (P < 0.001) after estradiol treatment. Growth stimulation by estradiol and TSC was blocked by the antiestrogen. Similar to other MCF-7 cell lines, the MCF-7 cells used in this study contain predominantly ER-α. Compared with ER-α, the amount of ER-β protein is not detectable by Western blot analysis using several antibodies, whereas the amount of ER-β mRNA is barely detectable using an RNase protection assay, suggesting that the effects of TSC are mediated by ER-α.

Effect of TSC treatment on the concentration of ER-α protein

To determine whether TSC has estrogen-like effects on the concentration of ER-α protein, an enzyme immunoassay was used. To verify the reproducibility of TSC preparations, three different TSC batches were used for the in vitro studies. Lot nos. 1 and 2 were obtained from Murty, and lot no. 3 was from Lovelace Respiratory Institute. MCF-7 cells were treated for 24 h with 10−9 m estradiol or 0.04–40 μg/ml TSC (Fig. 2). As expected, estradiol treatment resulted in an approximate 62% decrease (P < 0.001) in the concentration of

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We used the Kolmogorov-Smirnov test to check the normality assumption for our data, and from our tests, we have obtained P values > 0.05. We have also used the F test to determine the equal variance assumption and obtained insignificant P values. Therefore, we conclude that the normality assumption is valid, and we can conduct the Student’s t tests with equal variances. One-way ANOVA was used to test the overall difference between the mean values of treatment groups or experiments. The F test has been applied for different animal groups to test the null hypothesis that the mean values in all four groups are the same. After fitting the model, we obtained an F-statistic value of 148.86, with a corresponding P value < 0.001. Therefore, the difference between mean values is statistically significant. To control the inflation of the type I error, we applied the Bonferroni adjustment for P values, and the conclusions are drawn based on adjusted P values. We then performed a series of two-tailed unpaired Student’s t tests with equal variances to determine the statistical significance or differences in cell number, ER, and PR protein or mRNA, pS2 mRNA, C3 mRNA, or reporter gene activity among treatment groups compared with control. A P value < 0.05 was deemed significant (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).
ER-α. Increasing concentrations of TSC also resulted in a decreasing concentration in ER-α, from 415 fmol/mg protein in control cells to 328, 282, 162, and 154 fmol/mg protein after treatment with 0.04, 0.4, 4.0, or 40.0 μg/ml TSC, which represents a 21% (P < 0.05), 32% (P < 0.001), 61% (P < 0.001), and 63% (P < 0.001) decrease, respectively (Fig. 2A). The decrease in ER-α protein was not affected by addition of 10⁻⁹ M estradiol (Fig. 2B), and no variability was observed in experiments using different batches or sources of TSC (Fig. 2C; P > 0.05). All subsequent experiments, in which the TSC batch is not specified, were performed with lot no. 1.

Effect of TSC on the steady-state amount of ER-α mRNA

To determine whether the reduction in ER-α protein paralleled a reduction in the steady-state amount of ER-α mRNA, an RNase protection assay was performed. MCF-7 cells were treated with 10⁻⁹ M estradiol or 0.04–40 μg/ml TSC, and the effects of treatment on the steady-state amount of total ER-α mRNA were measured. The amount of ER-α mRNA was quantified by phosphor imaging and normalized to the amount of the acidic ribosomal phosphoprotein PO, 36B4 mRNA. The data are presented in Fig. 2 as percentage of control. Treatment with 10⁻⁹ M estradiol resulted in a 63% decrease in ER-α mRNA amounts (P < 0.05), which is in agreement with our previous observations (21). Treatment with 0.04, 0.04, 4.0, or 40.0 μg/ml TSC resulted in a 20% (P < 0.05), 29% (P < 0.05), 44% (P < 0.001), and 62% (P < 0.001) decrease in ER-α mRNA, respectively (Fig. 2A), and correlated with the magnitude of the effect on ER-α protein. A time course of the response to 40 μg/ml TSC is presented in Fig. 2D. TSC produced a rapid decrease in ER mRNA; by 3 h, a 52% decrease was observed (P < 0.001). The maximum decrease of approximately 62% was observed after 24-h treatment (P < 0.001).

Effect of TSC treatment on ER-α activity

To determine the effect of TSC on the amount of PR protein, an enzyme immunoassay was performed. MCF-7 cells were treated with 10⁻⁹ M estradiol or 40 μg/ml TSC in the presence or absence of the antiestrogen ICI 182,780 (5 × 10⁻⁷ M) for 24 h, and the concentration of PR protein was measured (Fig. 3A). In response to treatment with TSC, the PR concentration increased 5-fold when compared with control levels (P < 0.01). The magnitude of this increase was similar to the increase in PR concentration after treatment with 10⁻⁹ M estradiol. Treatment with 10⁻⁹ M estradiol resulted in a 6-fold increase (P < 0.01) in PR over control values. To determine if the effects of TSC were mediated by ER-α, the ability of the antiestrogen to block the effect of TSC was tested. As expected, ICI 182,780 blocked the effect of estradiol. The antiestrogen also blocked the effect of TSC, sug-
Increasing concentrations of TSC in- 

gested that the effects of the condensate on the expression of PR are mediated by ER. Treatment with TSC had a similar effect on PR mRNA. There was a 5-fold increase in PR mRNA (P < 0.001) that was blocked by the antiestrogen.

To establish whether TSC regulates other estrogen-responsive genes, MCF-7 cells were treated with 40 µg/ml TSC, and the amount of pS2 mRNA was measured by an RNase protection assay. TSC induced pS2 mRNA by 2-fold (P < 0.001) over control values (Fig. 3B). Estradiol, 10⁻⁹ M, induced a 2-fold increase in pS2 mRNA (P < 0.001). In the case of PR, the effect of TSC on pS2 mRNA was blocked by 5 × 10⁻⁷ M ICI 182,780, suggesting that the effects of TSC are mediated by ER.

To determine whether TSC activates ER-α, transient cotransfection assays were used. A wild-type ER-α expression vector and an estrogen response element-CAT reporter construct were cotransfected into COS-1 cells. The transfected cells were treated with 10⁻⁹ M estradiol or 40 µg/ml TSC, and CAT activity was measured in the presence or absence of the antiestrogen (Fig. 4A). Estradiol stimulated CAT activity by approximately 13.5-fold (P < 0.001). TSC produced an 11-fold increase (P < 0.05) in CAT activity that was blocked by the antiestrogen. Similar results were obtained when CHO cells were transiently cotransfected with the same wild-type ER-α and an estrogen response element-luciferase reporter (Fig. 4B). Estradiol and 40 µg/ml TSC (lot nos. 1–3) stimulated luciferase activity by about 6-fold (P < 0.0005 and P < 0.02, respectively). Increasing concentrations of TSC in-

creased ER-α activity (Fig. 4C). The increase in CAT activity was 6- (P < 0.05), 7.5- (P < 0.002), 11- (P < 0.05), and 8-fold (P < 0.05), respectively, upon treatment with TSC concentrations of 0.4, 4.0, 40.0, and 80.0 µg/ml, respectively.

AIB1 is a member of the steroid receptor coactivator family p160/SRC that is involved in the regulation of steroid receptor-mediated transcription. The splice variant, AIB-δ 3, is overexpressed in MCF-7 breast cancer cells and in breast tumor tissue (33), suggesting that the overexpression of this isoform may play a role in breast cancer. In this study the ability of AIB-δ 3 isoform to affect the transcriptional activity of TSC was determined. In the presence of AIB-δ 3, luciferase activity was increased to 17-fold upon estradiol and 23- to 26-fold upon TSC treatment (P < 0.001). These results suggest that similar to estrogens, TSC induction of transcription was much greater in the presence of AIB-δ3.

To identify the region of ER-α involved in activation by TSC, a chimeric receptor containing the hormone binding domain of ER-α was used. This chimera receptor consists of the DNA binding domain of the yeast transcription factor GAL-4 fused to the hormone binding domain of ER-α (GAL-ER). Stimulation of transcription by GAL-ER from a GAL-4-responsive CAT reporter gene requires estrogen. When the chimera receptor GAL-ER and the Gal-4-CAT reporter construct were transiently cotransfected into COS-1 cells and treated with 40 µg/ml TSC, there was a 3.5-fold increase in CAT activity (P < 0.01) compared with a 4-fold increase in activity upon treatment with estradiol (P < 0.01, Fig. 4D). The ability of TSC to activate GAL-ER suggests that TSC activates ER-α through the hormone binding domain.

**Activation of ER-α mutants by TSC**

We have previously identified amino acids C381, C447, E523, H524, and D538 as potential interaction sites of bivalent metals, with the hormone binding domain of ER-α and C381, C447, H524, K529 and/or K531, and N532 as potential sites of interaction with metals. To ask whether the metal/metalloid contaminants in TSC are responsible for the activation of ER-α, the ability of TSC to activate ER-α mutants C381A, C447A, E523A, H524A, N532D, and D538A, the double mutant K529Q K531Q, and the triple mutant K529Q K531Q N532D was tested. The ER-α mutants C417A and C530A were included as nonspecific controls. The mutants were transiently cotransfected with an estrogen response element-CAT construct into COS-1 cells, and the cells were treated with 10⁻⁹ M estradiol or 40 µg/ml TSC. The amount of CAT activity was measured, expressed as percent conversion to its acetylated forms, and normalized to the amount of β-galactosidase activity (Fig. 5). After treatment with estradiol, there was an approximate 10- to 23.5-fold increase in CAT activity with all the mutants (P < 0.05) except H524A. Hormone treatment of H524A resulted in 6-fold induction of CAT activity (P < 0.001). These results corroborate previous studies demonstrating the ability of estradiol to transactivate these mutants. Similar to estradiol, treatment of the cysteine mutants, C417A and C530A, with TSC resulted in an approximate 18- (P < 0.05) and 14-fold (P < 0.05) increase in CAT activity. In contrast to the effects observed with C417A and C530A, TSC only weakly activated the mutant C381A (2-fold; P < 0.001) and
did not activate C447A, suggesting that cysteines C381 and C447 may be involved in activation of ER-α by TSC. Mutation of E523, H524, K529 and K531, N532, or D538 also resulted in a substantial inhibition of activation of ER-α by TSC. Compared with the wild-type receptor, TSC activated only weakly the mutants E523A, H524A, N532D, and D538A, resulting in an approximate 6- (P < 0.05), 3- (P < 0.01), 5.5- (P < 0.001), and 3-fold (P < 0.001) increase in CAT activity, respectively. These results suggest that E523, H524, N532, D538, and at least one or both of the lysines K529 or K531 may also play a role in the interaction of TSC with ER-α. Together, these results suggest that the metal/metalloid contaminants in TSC may be responsible for the activation of ER-α through the formation of a complex with amino acids in the hormone binding domain of the receptor.

**Binding of TSC to the ER**

To determine whether TSC blocked estradiol binding to ER-α, the effect of TSC on hormone binding was measured using a single-dose ligand binding assay. Recombinant human ER-α was treated with various concentrations of TSC...
In the case of 0.04 μg/ml TSC, the presence of TSC, the dissociation constant of estradiol was then assayed by adding 10^{-8} M [3H]estradiol in the presence or absence of a 200-fold m excess of DES for 18 h at 4 °C. As shown in Fig. 6A, TSC blocked the binding of estradiol to the receptor. Hormone binding decreased with increasing TSC concentration. Maximal inhibition was seen after a preincubation with 400 μg/ml TSC.

To establish whether TSC altered the binding affinity of estradiol to the receptor, a multiple dose ligand binding assay was performed. Extracts from MCF-7 cells were incubated with 0.004–40 μg/ml TSC and various concentrations of [3H]estradiol (10^{-12} to 10^{-7} M) in the presence or absence of a 200-fold m excess of DES. The affinity and binding capacity of the receptor were determined according to the method of Scatchard (32) (Fig. 6, B and C). In the absence of TSC, estradiol bound to the receptor with an equilibrium dissociation constant (Kd) of 5.9 (±2.8) × 10^{-10} M (n = 3). In the presence of TSC, the dissociation constant of estradiol was unchanged, but the number of binding sites decreased. In the case of 0.04 μg/ml TSC, the Kd was 6.3 (±2.1) × 10^{-10} M, and the maximum binding was 76.5 ± 13% the control (n = 3). For a TSC concentration of 0.4 μg/ml, the Kd was 6.2 (±1.9) × 10^{-10} M, and the maximum binding 56.5 ± 2% the control (n = 3). For TSC 4 μg/ml, we measured a Kd of 6.8 (±2.7) × 10^{-10} M, and the maximum binding was 37 ± 1% the control (n = 3). Finally, the Kd was 2.2 (±1.5) × 10^{-10} M for TSC 40 μg/ml, and the maximum binding was 24 ± 11% the control (n = 3). These data demonstrate that TSC blocks the binding of estradiol to ER. To investigate further whether different TSC batches affect the binding affinity, the three lots were used (Fig. 6B). As in the case of ER protein regulation, no difference in the binding affinity or capacity was observed in this experiment (P > 0.05).

**In vivo effects of TSC**

We used an ovariectomized animal model to determine whether TSC has estrogen-like activity in vivo. Female Sprague Dawley rats were ovariectomized at 28 d of age, and the uteri were allowed to involute for 2 wk before treatment with either TSC or estradiol in the presence or absence of ICI 182,780. Animals received either an estradiol pellet (60 μg/kg b.w.d release pellet), a single ip injection of TSC (10 mg/kg bw), or two ip injections of TSC (10 mg/kg bw) administered over 2 consecutive days. The doses of TSC are equivalent to one sixth and one third the tobacco smoke intake from a single cigarette of an active smoker, respectively. The effects of TSC in the uterus and mammary gland were examined on d 5 after treatment. In TSC-treated animals, there was a 1.7- and 2.1-fold increase in uterine wet weight in the 10 and 20 mg/kg bw treatment groups, respectively (Table 2). In the estradiol-treated animals, there was a 9.3-fold increase in uterine wet weight. ANOVA showed a significant difference between control and TSC-treated animals (P < 0.01 and P < 0.001, respectively). There was no statistically significant difference between the 1 and 2-d treatments with TSC. Similar results were also observed when two different lots of TSC were administered over 2 d at a final dose of 20 mg/kg bw (2.1- and 1.7-fold increase, respectively; P < 0.001; data not shown). In addition to in-
increasing the wet weight of the uterus, TSC also induced the expression of two estrogen-regulated genes, PR and the complement factor C3 (Fig. 7). There was a 2-fold increase in PR mRNA \((P < 0.01)\) (Fig. 7A) and a 26-fold increase in C3 mRNA \((P < 0.001)\) (Fig. 7C). The increase in PR mRNA and C3 mRNA was blocked by the antiestrogen, suggesting that the effects of the condensate are mediated by ER. To determine whether exposure to environmental amounts of TSC also influenced the expression of estrogen-regulated genes in the mammary gland, the effects of TSC on PR and complement C3 mRNA were again measured. There was a 4.4-fold increase in PR mRNA \((P < 0.05)\) (Fig. 7B) and a 15-fold increase in C3 mRNA \((P < 0.001)\) in the mammary tissue (Fig. 7D). The increase in PR mRNA and C3 mRNA was blocked by the antiestrogen, providing additional evidence that the effects of TSC \textit{in vivo} are also mediated by ER.

### Discussion

Tobacco smoke is a complex mixture of more than 4000 organic and inorganic compounds. The inorganic components include several bivalent cations, such as cadmium, cobalt, copper, nickel, chromium, lead, mercury, and tin, and the metal/metalloid anions arsenite, selenite, and vanadate (for review, see Ref. 13). We have demonstrated that the latter components represent a new class of potent environmental estrogens, named metallo-estrogens (6–12). The goal of this study was to determine whether environmentally relevant amounts of TSC activate ER-\(\alpha\). The results presented here provide evidence that TSC has potent estrogen-like activity in cultured cells and ovariectomized animals, and that the estrogen-like activity of the condensate may be due to the presence of metal contaminants. In MCF-7 cells, TSC mimicked the effects of estrogens on cell growth and gene expression. These responses were blocked by an antiestrogen, suggesting that the effects of the condensate are mediated by ER. TSC activated wild-type ER-\(\alpha\) and a chimera containing the hormone binding domain of the receptor in transfection assays, and blocked the binding of estradiol to purified recombinant ER-\(\alpha\), suggesting that the condensate activates ER-\(\alpha\) through a direct interaction with the hormone binding domain. Consistent with the ability of metal/metalloid cations and anions to interact with amino acids containing a thiol group or a negative or positive charge, respectively, TSC only weakly activated ER-\(\alpha\) containing single-point mutations at amino acids C318, C447, E523, H524, N532, or D538, suggesting that the metal contaminants in TSC may be responsible, in part, for activation of the receptor. In a similar study, Meek and Finch (34) showed that TSC \((3–300 \mu g/ml)\)

### Table 2. Effect of TSC on the wet weight of uteri

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterus wet weight % bw</th>
<th>Fold increase</th>
<th>Animal bw (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.023 ± 0.001</td>
<td>1</td>
<td>244 ± 20</td>
</tr>
<tr>
<td>TSC ((1 \times))</td>
<td>0.039 ± 0.001</td>
<td>1.7(^{a})</td>
<td>229 ± 28</td>
</tr>
<tr>
<td>TSC ((2 \times))</td>
<td>0.048 ± 0.003</td>
<td>2.1(^{a})</td>
<td>248 ± 12</td>
</tr>
<tr>
<td>TSC ((2 \times)) + ICI</td>
<td>0.025 ± 0.007</td>
<td>1.1</td>
<td>238 ± 7</td>
</tr>
<tr>
<td>E(_2)</td>
<td>0.215 ± 0.053</td>
<td>9.3(^{a})</td>
<td>191 ± 10</td>
</tr>
<tr>
<td>E(_2) + ICI</td>
<td>0.0130 ± 0.005</td>
<td>6</td>
<td>197 ± 17</td>
</tr>
<tr>
<td>ICI</td>
<td>0.016 ± 0.004</td>
<td>0.7</td>
<td>249 ± 6</td>
</tr>
</tbody>
</table>

\(^{a}\) \(P < 0.001\).
activates the GAL-ER chimera in a dose-dependent manner and displaces estradiol from the ER in rat uterus. The present study further demonstrates that TSC activates ER in estrogen target organs of ovariectomized animals. The condensate increased uterine wet weight, and induced the expression of estrogen-regulated genes in the uterus and mammary gland. The effects of TSC were also blocked by an antiestrogen, suggesting that the in vivo effects of the condensate are mediated by ER.

The ER belongs to a superfamily of ligand-inducible transcription factors. Two distinct regions within the ER contribute to its transcriptional activity, the AF-1 domain located in the amino terminus and the ligand dependent AF-2 domain located in the carboxy-terminal hormone binding domain. The AF-1 and AF-2 domains regulate transcription both independently and synergistically, depending on the promoter and cell type (35). In the absence of hormone, the inactive receptor is complexed with a host of proteins, including heat shock proteins, which prevent it from interacting with the cellular transcription apparatus. Upon binding estradiol, the hormone binding domain of the receptor undergoes a conformational change that permits it to bind to coactivators and initiate transcription. Similar to other steroid receptors (36–42), the hormone binding domain of ER-α contains 12 α-helices (H1-H12) folded into a three-layered antiparallel α-helical sandwich. The central core layer contains three α-helices (H5/6, H9, and H10) sandwiched between two additional layers of helices composed of H1–4, H7, H8, and H11. The central core of the hormone binding domain is flanked by H12 (40). Upon hormone binding, several major structural changes are thought to occur, including the rotation of H3 and repositioning of H12 (36). The rotation of H3 and repositioning of H12 result in the formation of a shallow hydrophobic groove that constitutes the AF-2 domain, the binding site for steroid receptor coactivators. In previous studies (7–9, 11) we identified C381, C447, E523, H524, and D538 as potential interaction sites in the hormone binding domain, with bivalent cations and C381, C447, H524, N532, and at least one, and possibly two, lysines, K529 or K531, as potential interaction sites with the metal anions. Cysteines C381 and C447 are located on helices H4 and H8, respectively. Histidine H524 is located on helix H11. Lysines K529 and K531 and asparagine N532 are located in the loop between helices H11 and H12, and aspartic acid D538 is located at the loop-helix H12 interface. The interaction of metals with these amino acids is thought to reposition H12 and induce the rotation of H3. The inability of TSC to activate fully ER-α containing single-point mutations of these amino acids suggests that the metal contaminants in TSC may contribute to the activation of the ER. Although the results of this study suggest that the metals in TSC are responsible for activating ER, it is possible that the organic chemicals also contribute to activation of the receptor. However, most of the organic compounds in TSC do not bind to ER, bind with very low affinity [one of 4,000 to one of 10,000 of the binding affinity of estradiol and metallo-estrogens (7–9, 11)], or act as antiestrogens. The pesticides, p,p′-dichlorodiphenyl dichloroethylene, p,p′-dichlorodiphenyl trichloroethylene, and eldrin, have weak estrogenic activity, with binding affinities that are several orders of magnitude lower than the binding affinity of TSC (43), suggesting that the estrogen-like effects of the condensate are due to the presence of the metals/metalloids. Moreover, only 16–17% of the pesticides in tobacco are transferred into the mainstream smoke. In addition, the concentration of pesticides in cigarettes decreased by more than 98% in the 1990s, suggesting that it is unlikely that the estrogen-like effects of TSC are due to the organic chemicals but are due to the much stronger estrogenic activity of the metals/metalloids. In contrast to the pesticides, the polycyclic aromatic hydrocarbons, benzo(a)pyrene, benzenanthracene, and benzenanthracene diphenols, are weak antiestrogens that block the actions of estradiol (43). In addition to their effects on ER-α, polycyclic aromatic hydrocarbons bind with high affinity to the aryl hydrocarbon receptor (44), which interacts with the estrogen signaling pathway to inhibit estrogen-induced gene transcription (45–47). This may explain why TSC does not induce a large increase in the growth of MCF-7 cells (only 2-fold increase).

Significant amounts of metals and metalloids are found in tobacco and in mainstream and sidestream tobacco smoke (reviewed in Ref. 13). As a result of the high efficiency of pulmonary absorption, smokers have higher body burdens of metals than nonsmokers (15), and the body burden increases with increasing tobacco use. Nonsmokers whose spouses smoke, i.e., passive smokers, also have significantly higher levels of metals compared with nonsmokers whose spouses do not smoke. Interestingly, human mammary tissue contains high concentrations of metals and metalloids, including cadmium (48), arsenic, nickel, chromium, mercury, and lead (49), suggesting that these metals may be potential risk factors for breast cancer. In support of a link between breast cancer and metals, studies show that the concentrations of copper, cobalt, vanadium, and tin are significantly elevated in the serum and/or tumors of breast cancer patients (50–56). Moreover, the serum concentration of copper and ceruloplasmin (a copper-containing enzyme) is increased in patients with benign disease and breast tumors (53, 57). In breast tumors, the concentration of copper also varies with the stage of the disease; the highest concentrations are observed in advanced disease (56). Epidemiological studies show a marginally significant association between chromium and breast cancer in postmenopausal women (55) but a significant association between cadmium and the disease (58).

Although cigarette smoking increases the body burden of metals, there is no consistent association between smoking during adult life and breast cancer. However, smoking, if initiated early in life and continued for a long period, is linked to an increased risk of the disease (59–68). Several studies show an increased risk of breast cancer if smoking occurs before a first full-term pregnancy, specifically 5 yr or more before the pregnancy (63–68), if smoking occurs for a long duration, and if the number of cigarettes is high (63). Reports on the relationship between breast cancer and passive smoke are less numerous but suggest that being married to a smoker or being exposed to tobacco smoke increases the risk in women who never actively smoked (69–72). However, these findings were not confirmed in a large prospective study that examined the association between breast cancer mortality and exposure to tobacco smoke due to spousal...
smoking (73). There is also evidence to suggest that breast cancer risk from passive cigarette smoke may be dependent on phenotype (72, 74). Although additional studies are required to clarify the association between breast cancer risk and tobacco smoke, these studies suggest that long-term smoking beginning early in life, presumably during breast development, increases the risk of the disease. The mammary gland is unique in that it grows and develops throughout the lifetime of a female, and estrogens play a central role in the growth and development of the gland (for review, see Ref. 75). It has been suggested that early-life exposure to environmental estrogens may alter mammary gland development and, consequently, breast cancer risk. The results of this study suggest that the metallo-estrogens in tobacco smoke are good candidates for such a role. Although the link between cigarette smoking, metals/metalloids, and breast cancer remains to be defined, the ability of TSC to mimic the action remains to be defined, the ability of TSC to mimic the action of estrogen is, therefore, a potential risk factor for breast cancer.

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