Gene Induction Studies and Toxicity of Chemical Mixtures

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As part of its mixtures program, the Agency for Toxic Substances and Disease Registry (ATSDR) supports *in vitro* and limited *in vivo* toxicity testing to further our understanding of the toxicity and health effects of chemical mixtures. There are increasing concerns that environmental chemicals adversely affect the health of humans and wildlife. These concerns have been augmented by the realization that exposure to chemicals often occurs to mixtures of these chemicals that may exhibit complex synergistic or antagonistic interactions. To address such concerns, we have conducted two studies with techniques that are being used increasingly in experimental toxicology. In the first study, six organochlorine pesticides (4,4′-DDT, 4,4′-DDD, 4,4′-DDE, aldrin, dieldrin, or endrin) were selected from the ATSDR Comprehensive Environmental Response, Compensation and Liability Act of 1980 (or Superfund) priority list and tested for their ability to modulate transcriptional activation of an estrogen-responsive reporter gene in transfected HeLa cells. In these assays, HeLa cells cotransfected with an expression vector encoding estrogen receptor and an estrogen-responsive chloramphenicol acetyltransferase (CAT) reporter plasmid were dosed with and without selected environmental chemicals either individually or in defined combinations. Estradiol consistently elicited 10- to 23-fold dose-dependent inductions in this assay. By contrast, all six of the organochlorine pesticides showed no detectable dose-related response when tested either individually or in binary combinations. Thus, these chemicals as binary mixtures do not exhibit any additional estrogenicity at the levels tested in these assays. In the second study, arsenic [As(V)], cadmium [Cd(II)], chromium [Cr(III, VI)], and lead [Pb(II)] were tested in a commercially developed assay system, CAT–Tox (L), to identify metal-responsive promoters and to determine whether the pattern of gene expression changed with a mixture of these metals. This assay employs a battery of recombinant HepG2 cell lines to test the transcriptional activation capacity of xenobiotics in any of 13 different signal-transduction pathways. Singly, As(V), Cd(II), Cr(III, VI), and Pb(II) produced complex induction profiles in these assays. However, no evidence of synergistic activity was detected with a mixture of Cd(II), Cr(III), and Pb(II). These results have shown metal activation of gene expression through several previously unreported signal-transduction pathways and thus suggest new directions for future studies into their biochemical mechanisms of toxicity. In conclusion, the *in vitro* methods used in these studies provide insights into complex interactions that occur in cellular systems and could be used to identify biomarkers of exposure to other environmental chemical mixtures. *Key words:* aldrin, arsenic, cadmium, chemical mixtures, chromium, 4,4′-DDD, 4,4′-DDE, 4,4′-DDT, dieldrin, endrin, endocrine disruptors, estrogen, lead. *Environ Health Perspect* 110(suppl 6):947–956 (2002).
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Development of accurate risk assessment procedures and models is a complex, data-intensive task often impeded by a paucity of data. To promote the development of models and methods well founded on experimental research, a program is needed to ensure sufficient coordination between appropriate data generation and data use. Toward this goal, and as part of the mixtures program at the Agency for Toxic Substances and Disease Registry (ATSDR), well-designed, short-term experimental research studies are being supported to elucidate toxicologic mechanisms to better understand the molecular toxicology of chemicals, particularly their mechanisms of interaction, and to establish qualitative and quantitative models. The goal of the research component of the mixtures program is to bring together laboratory investigators, model developers, and risk assessors to ensure that experimental designs addressing existing data gaps will be employed.

Because humans are exposed to several chemicals and their combinations, toxicity testing has become the cornerstone of chemical hazard assessment. Although ideally it is desirable to test in whole-animal models, the number of environmental chemicals and their possible combinations are too large to be adequately evaluated in the test systems used in classic toxicity testing procedures and protocols. Hence, several *in vitro* assays are being used by the scientific community to screen for biologic activity, understand the mode and mechanisms of action of chemical toxicity in target organs, and estimate the joint toxicity of chemical mixtures. However, the results of *in vitro* studies should be carefully analyzed to determine the plausibility of biologic activity in the whole-animal systems, taking into consideration the biologic and pharmacokinetic processes important to the *in vivo* expression of toxicity.

For about a decade, ATSDR has supported the use of alternative methods for laboratory testing and the development of computational tools to augment knowledge in the areas of hazard identification and toxicity evaluation (*1*). In 1994 ATSDR hosted an international symposium of experts in computational methods that led to the establishment of a state-of-the art computational toxicology laboratory (*2*). One of the activities of this laboratory is the agency's mixtures program, which consists of identification of environmental mixtures, joint toxicity assessment, and experimental testing (*3*). Through the mixtures program, the agency has supported *in vitro* and limited *in vivo* toxicity testing targeted to fill data gaps needed to support physiologically based pharmacokinetic modeling designed to improve our understanding of the toxicity and health effects of chemical mixtures (*1*).

In this article, we review results from two different studies conducted to evaluate the biologic activity of persistent pollutants identified in environmental media at hazardous waste sites. The first study measured the ability of six high-priority persistent organochlorine pesticides and defined mixtures to modulate transcriptional activation of an estrogen-responsive reporter gene in transfected HeLa cells. In the second study, four high-priority metals and a mixture were tested for their ability to induce 13 different gene promoters in a battery of recombinant HepG2 cells (Table 1). The organic pollutants and their Comprehensive Environmental Response, Compensation and Liability Act of 1980 (or Superfund) (Public Law 96510, 11 December 1980) priority rankings (indicated in square brackets) are *p,p′*-DDT [13], *p,p′*-DDD [26], *p,p′*-DDE [22], aldrin [25], dieldrin [18], and endrin [39]. The metals studied were arsenic (As) [1], lead (Pb) [2], cadmium (Cd) [7], Cr(VI) [16], and Cr(III) [69]. The ranking of these chemicals is

determined on the basis of their frequency of occurrence, toxicity, and potential for human exposure (4).

Materials and Methods

Experimental Procedures

Chemicals used in these studies, their CAS numbers, and purity are given in Table 2. Details of the experiments and procedures have been published (5,6). Briefly, in the first series of experiments, dosing solutions were prepared by dissolving the neat chemicals in 95% ethanol followed by 10-fold serial dilutions. The concentration of ethanol was kept constant at all doses of pesticides, so the cells were consistently exposed only to 0.1% ethanol. HeLa cells, a transformed human ovarian carcinoma cell line, were seeded (~6 × 10^5 cells/well) in 6-well microtiter plates, approximately 40–60% confluent at the time of seeding. The HeLa cell transfection system used in these assays employed an estrogen-responsive chloramphenicol acetyl-transferase (CAT) reporter vector regulated by a promoter containing two contiguous copies of an estrogen response element. These assays were previously shown to give a robust response to 17β-estradiol (E_2) and to give measurable responses to weak environmental estrogens such as 2-2-bis(p-hydroxyphenyl)-1,1,1-trichloroethylene, nonylphenol, and o,p′-DDT (7). The duration of chemical exposures was 18 hr in all cases, and a broad range of pesticide doses (0, 0.001, 0.01, 0.1, 1.0, and 10 μM) was chosen to mimic serum levels measured in previous experiments with mice and for comparability with earlier work using this assay system (7) and other published reports (8,9). Triplicate cell cultures were dosed at each concentration of hormone, pesticide, or pesticide mixture, and the cells were harvested 18 hr after dosing. After

cell harvesting, detergent cell lysates were either quick-frozen at –80°C, or aliquots were assayed immediately for CAT protein using the CAT–ELISA (enzyme-linked immuno-sorbent assay) kit (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's instructions. Additional aliquots of each cell lysate were subsequently assayed to determine total protein concentration using the BioRad Protein Assay Reagent (BioRad, Hercules, CA, USA) (10). The amount of CAT protein measured in the CAT–ELISA assay for each cell lysate was normalized to that well's amount of total protein, and the results were tabulated as nanograms CAT per milligram protein.

In the second series of experiments, appropriate amounts of metal salt were added to the 1 mM humic acid (HA) stock to obtain the desired metal concentration. The HA or metal–HA stocks were then diluted 10-fold into cell culture medium, which was serially diluted to obtain the experimental doses chosen on the basis of preliminary

dose–range-finding experiments. HepG2 cells, a human hepatoma cell line, and the 13 recombinant cell lines derived from them (Xenometrix, Boulder, CO, USA) (11) were seeded (~5.5 × 10^4 cells/well) in all wells of a single row of a 96-well microtiter plate, one row per recombinant cell line; the cells were approximately 40–60% confluent at the time of seeding. Cell viability was assayed on each plate at each dose using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (12). Thus, two microtiter plates were required to accommodate all 13 recombinant cell lines plus the wild-type HepG2 cell line while allowing two columns per treatment. Although this configuration comprises a single CAT–Tox (L) assay, these experiments were always done in triplicate using three such pairs of microtiter plates. Additionally, independent replicates of each experiment were performed on different dates. Cells were incubated at 37°C and 5% CO_2 for 48–72 hr before exposure to chemical

Table 2. Organochlorine pesticides and inorganic chemicals used in these studies.

Common or trade name	Chemical name	CAS no.	Purity (%)
Estradiol	17β-Estradiol	50-28-2	99+
p,p′-DDT	Bis(p-chlorophenyl)-2,2,2-trichloroethane	50-29-3	98
p,p′-DDD	Dichlorodiphenyldichloroethane	72-54-8	99
p,p′-DDE	Dichlorodiphenyldichloroethylene	72-55-9	99
Aldrin	1,4:5,8-Dimethanonaphthalene, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-, (1a,4a,4ab,5a,8a,8ab)-	309-00-2	98
Dieldrin	2,7:3,6-Dimethanonaphth[2,3-b]oxirene, 3,4,5,6,9,9-hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-, (1aa,2b,2aa,3b,6b,6aa,7b,7aa)-	60-57-1	98
Endrin	2,7:3,6-Dimethanonaphth[2,3-b]oxirene, 3,4,5,6,9,9-hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-, (1aa,2b,2ab,3a,6a,6ab,7b,7aa)-	72-20-8	98
Arsenic (V)	Ammonium arsenate	7784-44-3	95+
Cadmium (II)	Cadmium acetate dihydrate	5643-04-4	98
Chromium (VI)	Potassium dichromate	7778-50-9	99
Chromium (III)	Chromium acetate	1066-30-4	99
Lead (II)	Lead nitrate	18256-98-9	99+
Humic acid		1415-93-6	Technical grade

Table 1. Different promoters and their functional definitions.

Abbreviation	Gene promoter name	Function	Implications
CYP 1A1	Cytochrome P450 1A1	Ah receptor–responsive promoter (35,36)	Aryl hydrocarbons
GST Ya	Glutathione S-transferase Ya	Contains both antioxidant and Ah response elements (30,31)	Aryl hydrocarbons; oxidative stress
XRE	Xenobiotic response element	Two contiguous oligonucleotide copies of Ah receptor binding site from the Cyp 1A1 promoter (36)	Aryl hydrocarbons
hMTIIA	Human metallothionein II A	Heavy metal–responsive promoter (26, 27)	Heavy metal stress
FOS	c-fos immediate early oncogene	Responds to mitogens and DNA damage (32)	DNA damage; oxidative stress
NFκBRE	Nuclear factor kappa B response element	Immune inflammatory and acute phase response (34,53)	Inflammatory response; oxidative stress
HSP70	70-kDa Heat shock protein	Induced by heat, heavy metals, protein denaturants (54,55)	Stress response
CRE	Cyclic AMP response element	Cyclic AMP receptor–responsive promoter (65)	cAMP
p53RE	Tumor suppressor p53 response element	Responds to DNA damage (37,39)	DNA damage; oxidative stress
RARE	Retinoic acid response element	Responds to retinoic acid and analogs (67)	Retinoic acid
GADD153	153-kDa Growth arrest and DNA damage	Responds to DNA damage (40,44)	DNA damage
GADD45	45-kDa Growth arrest and DNA damage	Responds to DNA damage (64)	DNA damage
GRP78	78-kDa Glucose-regulated protein	Responds to protein deanaturation and DNA damage (43)	Protein denaturation;DNA damage

treatments. The duration of chemical exposures was 48 hr. In all experiments where cells were treated with Cr(VI) in HA, special care was taken to use freshly prepared stocks, because HA reportedly causes chemical reduction of Cr(VI) to Cr(III) (13,14). Specific details of chemical treatments for each metal or mixture are given in Table 3.

Because there has been increasing concern about the potential for synergistic increases in toxicity stemming from exposure to mixtures of chemicals, and because mixtures of these metals commonly occur as pollutants at hazardous waste sites on the U.S. Environmental Protection Agency's National Priorities List (15), we wanted to test combinations of some of these metals in the CAT–Tox (L) assays. To increase the likelihood of detecting potential synergistic effects, we decreased the top concentration of Cd(II) in the mixture to 7.5 μM, half the high dose used when testing Cd alone, so the high dose of the mixture contained 7.5 μM Cd(II), 750 μM Cr(III), and 100 μM Pb(II) in 100 μM HA.

After the treatment period, all the cells, except one row containing the wild-type HepG2 cell line reserved for cell viability assays, were lysed with 100 μL detergent lysis buffer. Ten-microliter pooled lysate aliquots from each pair of identically dosed cells were assayed to determine total protein concentration using BioRad Protein Assay Reagent (BioRad) according to the manufacturer's instructions (10). The amount of CAT protein in each lysate was then determined using a sandwich ELISA assay with CAT–ELISA reagents from Boehringer-Mannheim, as previously described (11). Transcriptional activities were calculated by dividing the amount of specific CAT protein measured in the CAT–ELISA assay for each cell lysate by the corresponding amount of total protein.

Data Analysis

In the first series of experiments, the mean and standard error were calculated for the amount of CAT protein measured in three independent experiments, each containing triplicate measurements at each dose; the results were plotted as bar graphs. Data were further analyzed with Student t-test, and the significance level was ascertained at $p < 0.05$.

For the second series of experiments, after logarithmic transformation of the transcriptional activities to stabilize their variances, a separate analysis of variance (ANOVA) (16) was performed for each combination of chemical and cell line. Within each ANOVA, one-sided and two-sided Dunnett's tests (17,18) were used to assess which treatment groups showed transcriptional activity significantly different from the control group at the α = 0.05 level. The ratio of the transcriptional activity for a dose group versus the control group, i.e., [CAT] (test sample)/[CAT] (zero dose control), is called the fold induction, which represents the dose-specific increase in activity induced by the treatment relative to baseline. In each experiment, the fold inductions for the three replicates were averaged. The means of these averages for data from experiments performed on different dates were calculated and plotted in the figures as bars, with their standard errors shown by lines extending above the bars.

Results

Figure 1A shows the results of testing p,p'-DDT, p,p'-DDD, and p,p'-DDE in individual assays. Estradiol produced a dose-responsive transcriptional induction profile in which 0.01 nM E_2 yielded approximately 3-fold induction over the vehicle control, 0.1 nM E_2 gave a 4-fold induction, and 1.0 nM E_2 gave maximal induction of approximately 10-fold. In contrast, none of the three DDT isomers— p,p'-DDT, p,p'-DDD, or p,p'-DDE—showed appreciable induction over the background levels of the vehicle controls at any of the five doses tested. The small apparent increases in
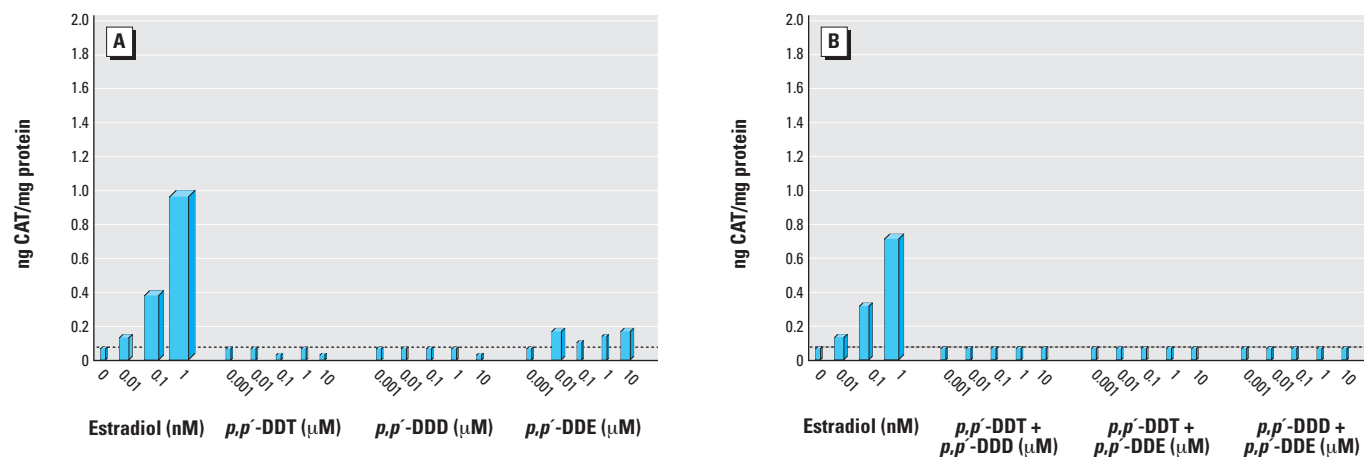
Table 3. Final doses of metals and metal mixtures used.

		Single chemical concentrations (μM)			
HA	As(V)	Pb(II)	Cd(II)	Cr(III)	Cr(VI)
0	0	0	0	0	0
6.25	50	6.25	1.25	50	0.625
12.5	100	12.5	2.5	150	1.25
25	150	25	5	250	2.5
50	200	50	10	500	5.0
100	250	100	15	750	10
		Mixture component concentrations (μM)			
Percent		Cd(II)		Pb(II)	Cr(III)
0		0		0	0
0.63		0.47		6.25	47
1.25		0.94		12.5	94
2.5		1.87		25	187
5.0		3.75		50	375
10.0		7.50		100	750

Figure 1. Transcriptional activation of an estrogen-responsive CAT reporter gene by p,p'-DDT, p,p'-DDE, and p,p'-DDD. HeLa cells cotransfected with the estrogen receptor expression vector pRSVmER and an estrogen-responsive CAT reporter plasmid, pERET81CAT, were dosed for 18 hr with vehicle, with 0.01–1.0 nM 17β-estradiol, or with 0–10 μM p,p'-DDT, p,p'-DDD, or p,p'-DDE (A). In parallel experiments, transfected cells were dosed with vehicle, with 0.01–1.0 nM 17β-estradiol, or with equimolar mixtures of p,p'-DDT + p,p'-DDD, p,p'-DDT + p,p'-DDE, or p,p'-DDD + p,p'-DDE (B). The results shown represent the mean of three independent experiments.

transcriptional activation of the estrogen-responsive CAT reporter shown by *p,p′*-DDE were not statistically significant and not particularly dose responsive. Although the apparent decrease in transcriptional induction below background levels seen with 10 μM *p,p′*-DDD was significant at the α = 0.05 level, this decrease almost certainly related to the high degree of toxicity of this compound, where visual inspection revealed that 50–70% of the cells were killed at the highest dose. A smaller decrease in transcriptional activation at 0.1 μM *p,p′*-DDT was not statistically significant, and no decrease in cell number was found at this or any other dose of *p,p′*-DDT. Thus, none of the three DDT isomers tested individually showed any significant evidence of estrogenicity in these assays.

Similar results were obtained when the three DDT isomers were tested in equimolar binary combinations, as shown in Figure 1B. In the combination assays, the pesticide concentration shown represents the sum of the concentrations of the two components of the mixture, e.g., 10 μM *p,p′*-DDT + *p,p′*-DDD (equimolar) means 5 μM *p,p′*-DDT + 5 μM *p,p′*-DDD. None of the combinations—*p,p′*-DDT + *p,p′*-DDD, *p,p′*-DDT + *p,p′*-DDE, or *p,p′*-DDD + *p,p′*-DDE— showed any significant estrogenic activity above the vehicle control background levels. This was true, despite the fact the estradiol-positive controls in these experiments yielded a robust induction profile, showing that these cells were fully capable of responding to an estrogenic stimulus. Again, where *p,p′*-DDD was present in the mixture, increased toxicity and noticeable cell loss were observed at the highest dose, but the small decrease in CAT production measured in this case was not statistically significant.

The results of testing aldrin, dieldrin, and endrin are shown in Figure 2. Figure 2A shows the results of testing these three pesticides individually. The estradiol-positive controls again produced dose-responsive transcriptional responses. However, neither aldrin, dieldrin, nor endrin showed any significant estrogenic activity at any of the five doses tested. This occurred in experiments where the estradiol-positive controls gave dose-responsive inductions up to 16-fold at 1 nM estradiol.

When aldrin, dieldrin, and endrin were tested in equimolar binary combinations (Figure 2B), no activity above the zero-dose controls was seen for any of the three mixtures. Thus, these three cyclodiene pesticides showed no evidence of estrogenic activity individually, and clearly showed no synergistic enhancement of estrogenic activity in any of the combinations tested.

In the second study (Figure 3), HA alone produced a moderate, dose-dependent
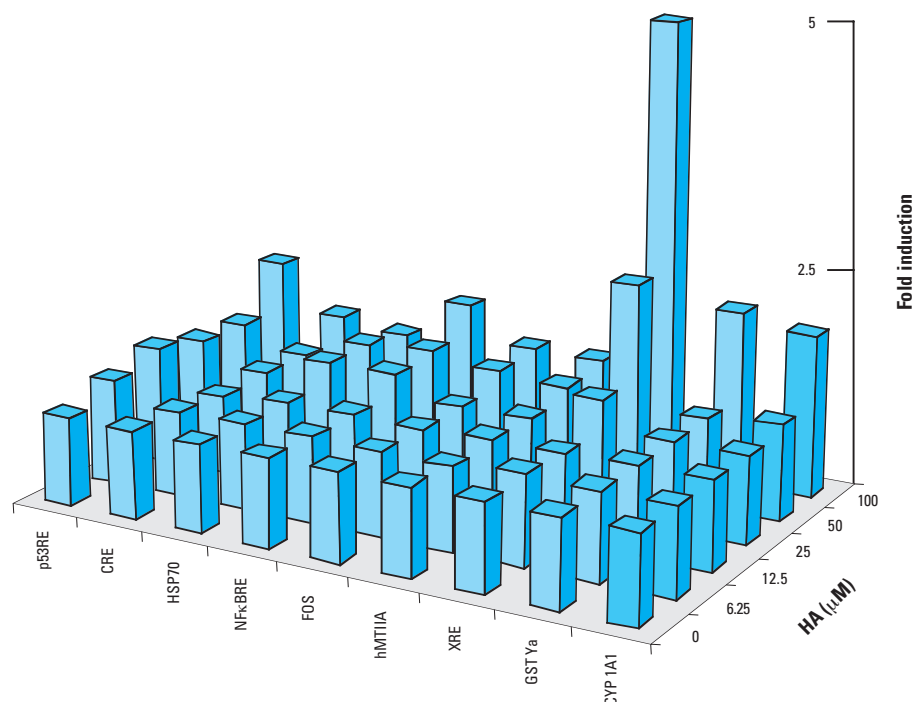

Figure 3. Profile of transcriptional induction of selected stress-response promoters in transgenic HepG2 cells dosed for 48 hr with 0–100 μM HA. The results shown represent the mean of four independent experiments for which fold inductions from three replicate assays were averaged. See Table 3 for details of dosing.
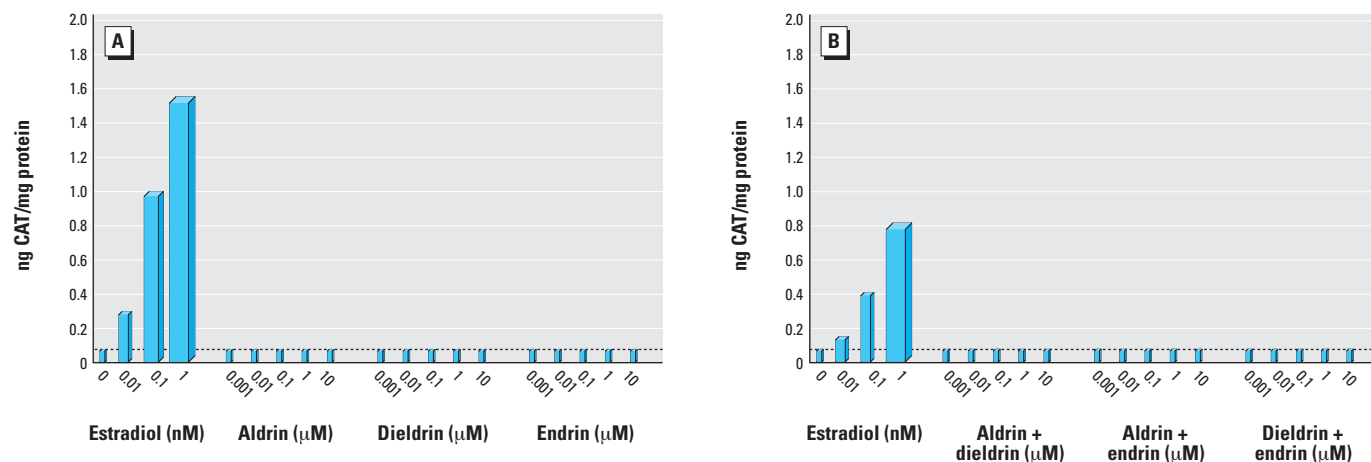

Figure 2. Transcriptional activation of an estrogen-responsive CAT reporter by aldrin, dieldrin, and endrin. Twenty-four hours after cotransfection with the estrogen receptor expression vector pRSVmER and the estrogen-responsive CAT reporter plasmid pERET81CAT, HeLa cells were dosed for 18 hr with vehicle, with 0.01–1.0 nM 17β-estradiol, or with 0–10 μM aldrin, dieldrin, or endrin (*A*). In parallel experiments (control), transfected cells were dosed with vehicle, with 0.01–1.0 nM 17β-estradiol, or with equimolar mixtures of aldrin + dieldrin, aldrin + endrin, or dieldrin + endrin (*B*). The results shown represent the mean of three independent experiments.
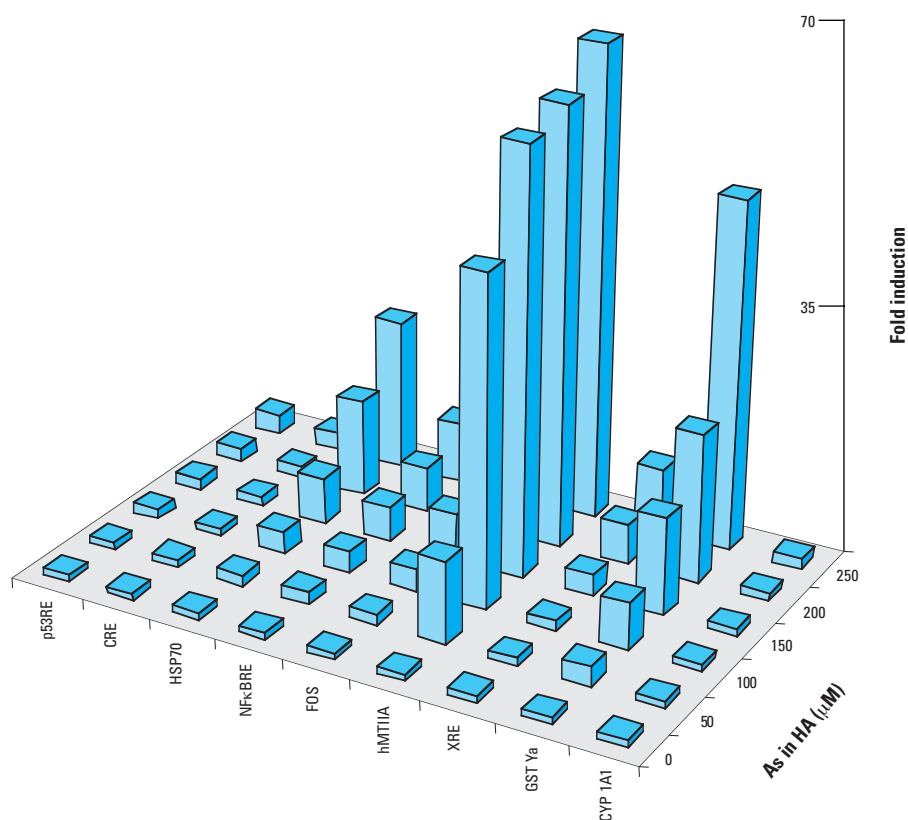
Figure 4. Transcriptional induction profile in transgenic HepG2 cells dosed with 0–250 μM ammonium arsenate in HA for 48 hr. The results shown represent the mean of three independent experiments for which fold inductions from three replicate assays were averaged. See Table 3 for details of dosing.
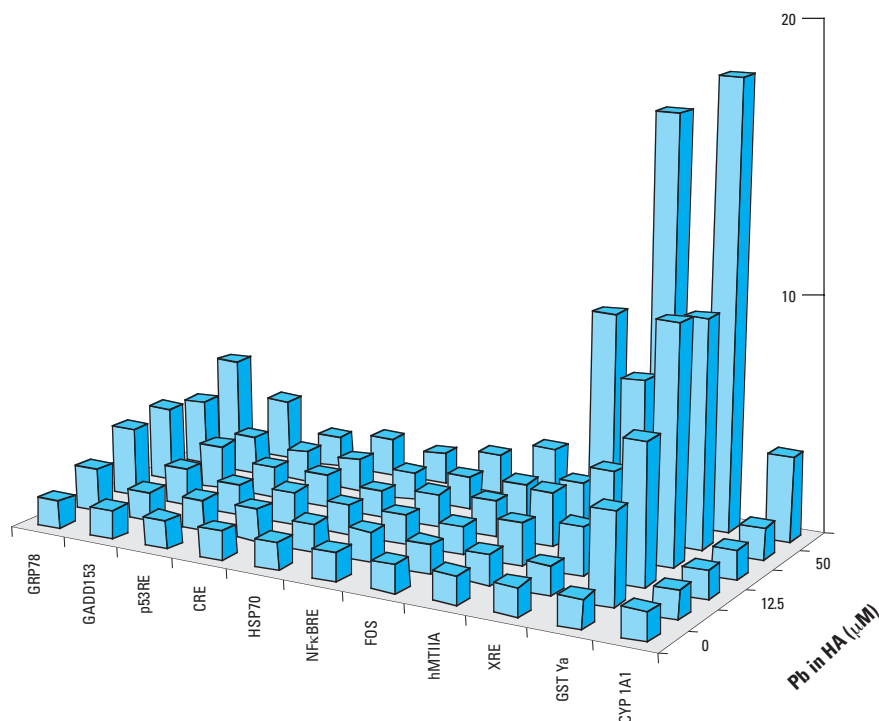

Figure 5. Profile of transcriptional induction for transgenic HepG2 cells dosed with 0–100 μM Pb(II) in HA for 48 hr. The results shown represent the mean of three independent experiments for which fold inductions from three replicate assays were averaged. See Table 3 for details of dosing.

induction of the xenobiotic response element (XRE) promoter up to 5-fold at the highest concentration (100 μM). HA-induced increases in transcription of the cytochrome P450 1A1 (CYP1A1), glutathione S-transferase Ya (GST Ya), and tumor suppressor p53 response element (p53RE) promoters were all 2-fold or less and were significant only at the highest dose. HA had relatively little effect on cell viability at all doses tested (6). These results show that HA could be employed as a carrier for the metals without either seriously compromising the integrity of the cultured cells or producing a confounding pattern of reporter gene inductions.

As(V) in HA (Figure 4) produced a complex profile of inductions among the nine promoters. There was consistently strong dose-dependent induction of the human metallothionein II A (hMTIIA) promoter at all doses tested, up to 70-fold at the highest dose (250 μM). As also showed a strong dose-dependent induction of the 70-kDa heat shock protein (HSP70) promoter up to 23-fold at the high dose (250 μM). Strong transcriptional activation of the GST Ya promoter, with an average 52-fold increase in the high-dose group (250 μM) was observed; however, the fold induction values varied greatly. In addition to the two signature inductions, hMTIIA and HSP70, As also induced expression of the c-fos immediate early oncogene (FOS), XRE, and nuclear factor kappa B response element (NFκBRE) promoters at more moderate, but still dose-dependent levels, up to 12-, 10-, and 9-fold, respectively, at the high dose (250 μM). As produced a nearly linear decrease in cell viability over the range of doses tested, with viability decreasing to 69% at the highest dose (6).

Figure 5 shows that Pb(II) in HA induced the GST Ya promoter most strongly, with dose-related responses up to 16-fold at the highest dose (100 μM). However, as with As(V), these responses were highly variable. Less variability was seen in the responses from the XRE promoter, which gave a dose-dependent profile of inductions of similar magnitude, ranging up to 14-fold at the highest dose. Pb also caused moderate dose-related induction of the hMTIIA promoter up to 7-fold and smaller though still dose-related inductions of the 78-kDa glucose-regulated protein (GRP 78) and CYP1A1 promoters (3-fold) at the high dose. Pb had only moderate effects on cell viability, decreasing cell viability to 84% at the highest dose (6).

Cd in HA (Figure 6) showed greatest induction of hMTIIA, with dose-responsive increases throughout the range of doses tested (1–15 μM) up to 77-fold at the highest dose. Cd induction of gene expression from the GST Ya promoter was more moderate than

with As, giving induction values up to 14-fold at the highest dose (15 µM) but again was highly variable among the three independent experiments. Unlike As, however, Cd produced only moderate dose-dependent inductions of the HSP70 promoter, up to only 4-fold at the highest dose (15 µM), but gave a more striking profile of inductions of the XRE promoter, up to 32-fold at the high dose. In further contrast to As, Cd also caused strong dose-dependent induction of the CYP 1A1 promoter, up to 15-fold, but gave only small inductions of the NFκBRE and FOS promoters, up to 4- and 3-fold, respectively, at the high dose (15 µM). Cd produced a nearly linear decrease in cell viability down to 62% at the highest dose (6).

In marked contrast to the other metals tested, Cr(III) in HA failed to produce striking inductions among any of the promoters (Figure 7). This was true despite the use of very high doses of Cr(III) up to 750 µM. It is also noteworthy that, in contrast to the As, Pb, and Cd results, GST Ya was only slightly induced. The very small fold induction values seen for the cyclic AMP response element (CRE) (3- fold), FOS (2-fold), 153-kDa growth arrest and DNA damage (GADD153) (2-fold), and XRE (2-fold) promoters were significant only at the highest (750 µM) dose and showed no indication of dose dependence at lower doses. Cr(III) did cause an approximately linear decrease in cell viability down to 62% at the highest dose (6). Cr(VI), however, produced a strikingly different induction profile (Figure 8). At 10 µM, Cr(VI) produced greater than 2-fold induction of all the promoters assayed, although several promoter inductions were significant only at the highest dose [CYP1A1, GST Ya, hMTIIA, NFκBRE, GADD153, and GRP78]. The most striking dose-dependent inductions were the p53RE and FOS promoters: up to 44- and 38-fold, respectively, at the high dose. In addition to these two prominent responses, Cr(VI) also produced dose-related fold inductions of the XRE (13-fold), 45-kDa growth arrest and DNA damage (GADD45) (10-fold), HSP70 (8-fold), and CRE (7-fold) promoters, as well as inductions of NFκBRE (13-fold), CYP1A1 (8-fold), GADD153 (7-fold), and GST Ya (6-fold) that were significant only at the highest dose. Cr(VI) was highly cytotoxic, and at doses nearly two orders of magnitude lower than were used with Cr(III) caused a very sharp decline in cell viability (6). At any dose greater than 10 µM, essentially all the cells were killed.

The only prominent response from treating the cells with a mixture of 7.5 µM Cd(II), 750 µM Cr(III), and 100 µM Pb(II) in 100 µM HA was associated with the hMTIIA promoter, which showed a dose-related profile with a 50-fold induction at the highest dose
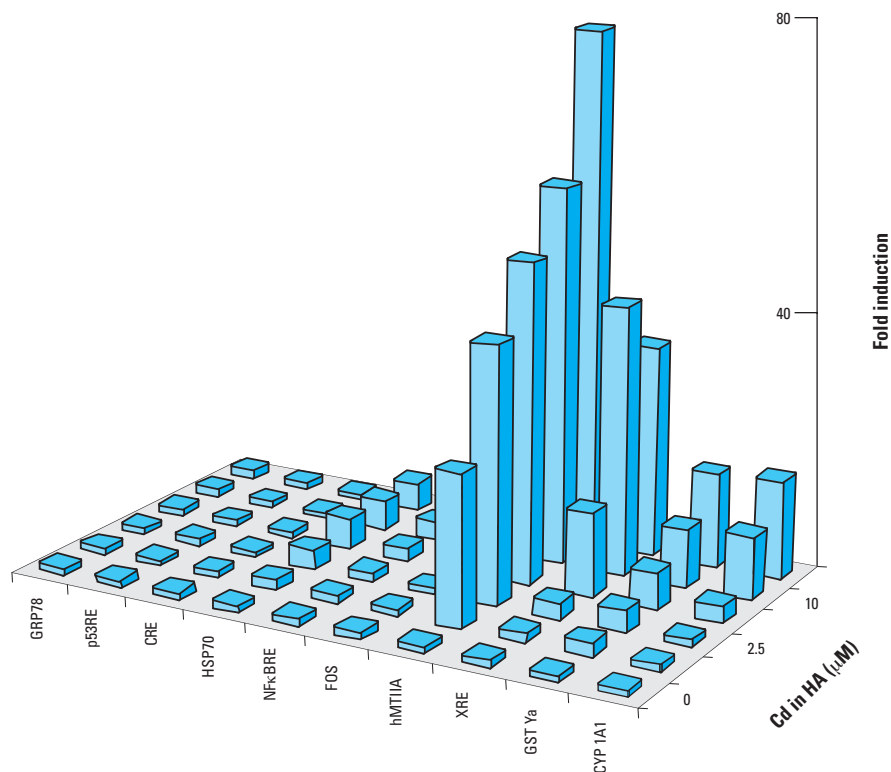

Figure 6. Profile of transcriptional induction and cell viability for transgenic HepG2 cells dosed with 0–15 µM Cd(II) in HA for 48 hr. The results shown represent the mean of three independent experiments for which fold inductions from three replicate assays were averaged. See Table 3 for details of dosing.
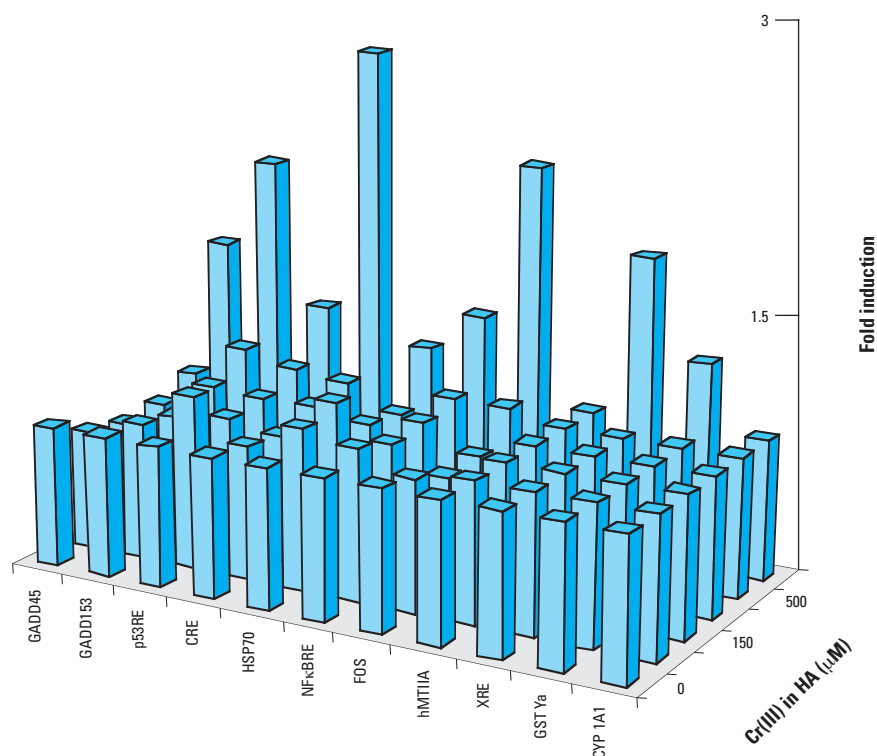

Figure 7. Profile of transcriptional induction for transgenic HepG2 cells dosed with 0–750 µM Cr(III) acetate in HA for 48 hr. The results shown represent the mean of three independent experiments for which fold inductions from three replicate assays were averaged. See Table 3 for details of dosing.
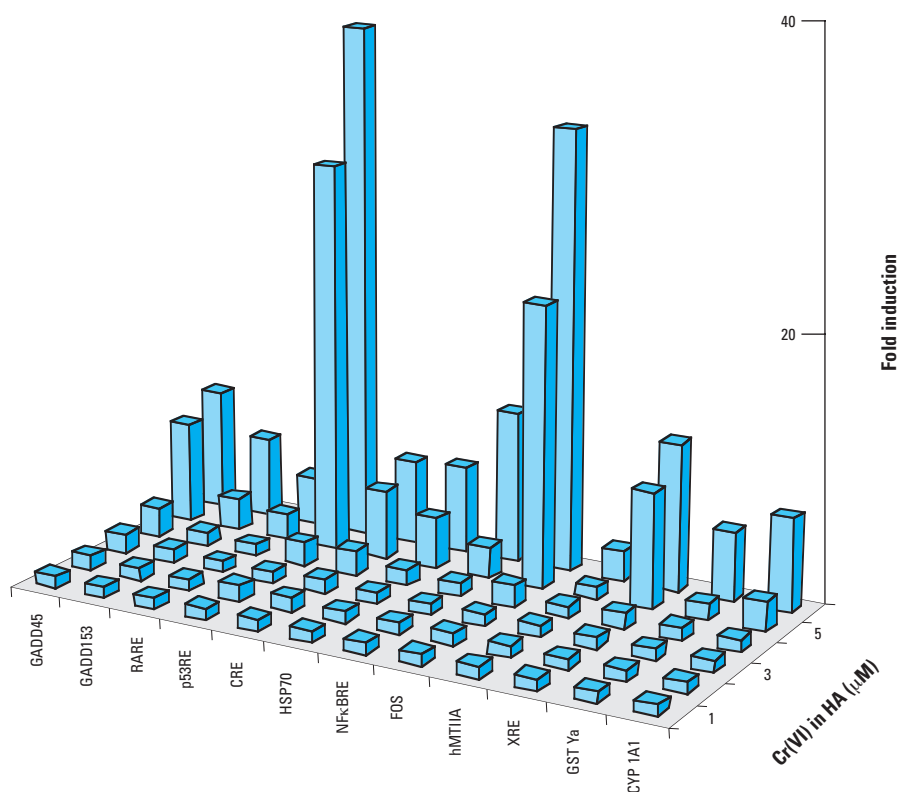
Figure 8. Profile of transcriptional induction for transgenic HepG2 cells dosed with 0–10 µM Cr(VI) in HA for 48 hr. The results shown represent the mean of two independent experiments for which fold inductions from three replicate assays were averaged. See Table 3 for details of dosing.
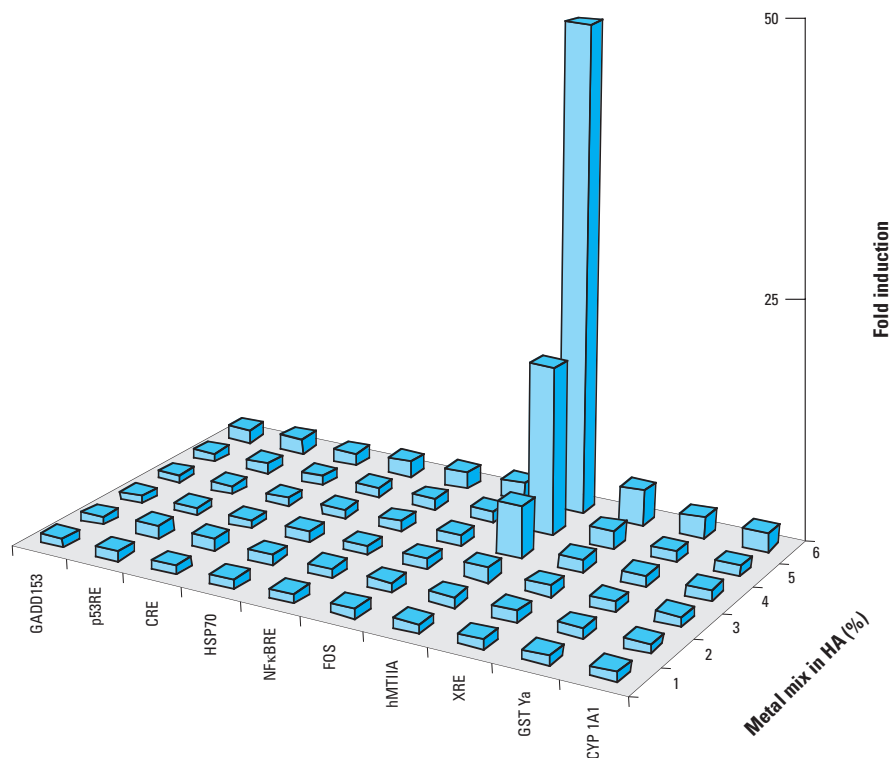

Figure 9. Profile of transcriptional induction for transgenic HepG2 cells dosed for 48 hr with 0–10% of a mixture containing Cd(II), Cr(III), and PB(II) in 1.0 mM HA. The results shown represent the mean of two independent experiments for which fold inductions from three replicate assays were averaged. The details of dosing are given in Table 3.

(Figure 9). There was a small, apparently dose-related induction of the XRE promoter (4-fold) at the high dose but only the high dose was significant. There were also small fold inductions (2-fold) of the CYP1A1, FOS, NFκBRE, HSP70, and p53RE promoters that were statistically significant only at the high dose. The apparent induction of the GST Ya promoter (2-fold) was not statistically significant. This mixture of metals had no appreciable effect on the viability of the cells across the range of doses tested (6).

Discussion

Existing literature offers mixed reports regarding possible estrogenic activity of the pesticides studied. p,p'-DDT has been reported to be weakly estrogenic in various *in vitro* assays (19–21). However, the widely reported estrogenic activity of technical-grade DDT most likely derives from its lesser component o,p'-DDT, whereas other studies suggest possible species differences in the estrogenicity of DDT (8). Its two metabolites, p,p'-DDD and p,p'-DDE have been reported to exhibit different effects in different model systems (20,22). However, consistent with our results, the bulk of evidence suggests that p,p'-DDE is nonestrogenic or very weakly estrogenic.

There are also mixed reports of endocrine activity for the cyclodiene pesticides. The potential estrogenic activity of aldrin remains unclear. It has been reported to yield uterotrophic effects and increased endometrial proliferation at very high doses in some studies but in others showed no detectable uterotrophic activity even at 1,000 mg/kg (23,24). Similarly, dieldrin has been reported to be weakly estrogenic (19) or nonuterotrophic alone (24) or in combination with endosulfan (25). Additionally, dieldrin alone or mixed with toxaphene did not induce significant increases in any of three estrogen-responsive end points (9). Thus, in addition to our findings, a sizable body of evidence suggests that dieldrin is not estrogenic. No previous reports assessing the potential estrogenic activity of endrin have been found.

Thus, there was virtually no detectable estrogenic activity for any of the six organochlorines tested singly. However, in each experiment, the estradiol-positive controls consistently produced dose-responsive CAT induction profiles. Given the essentially undetectable levels of activity shown by these pesticides tested individually, it is not surprising that estrogenic activity was not detectable with their equimolar binary combinations. Furthermore, it is clear that there were no synergistic interactions among combinations of these pesticides.

The results with As(V) agree with earlier reports demonstrating that the hMTIIA and

HSP70 promoters are characteristically induced by metals, including zinc, Cd, and As (*26–29*). The prominent, though variable, induction of the GST Ya promoter by arsenate in HA is a novel finding. The GST Ya promoter contains an antioxidant-response element in addition to an XRE, and its induction may signal a cellular response to oxidative stress (*30,31*). This possibility is substantiated by coordinate induction of the FOS and NFκBRE promoters, which are also associated with oxidative stress responses (*32–34*). The dose-dependent induction of the XRE-regulated reporter may be partly attributable to a low level of aryl hydrocarbons in the HA carrier (*35,36*). However, the levels of induction from this promoter were nearly 2-fold higher after treatment with arsenate in HA than those seen with HA alone (Figure 3). This suggests that arsenate has a direct or indirect effect on the XRE promoter. Finally, the results showing the coordinate dose-responsive induction of p53RE and GADD153 along with FOS, discussed previously, is strongly indicative of a cellular response both to DNA damage and to oxidative stress (*37–40*). Thus, arsenate exposure subjects these cells to toxic stresses involving an array of responses acting through several different and previously unreported signal-transduction pathways, including GST Ya, FOS, XRE, NFκBRE, GADD153, p53RE, and CRE. Knowledge of these additional signal-transduction pathways may offer new insights into the biochemical mechanisms of the toxicology and carcinogenicity of As.

The moderate induction of the hMTIIA and GRP78 promoters by Pb(II) (Figure 5) is consistent with earlier reports (*41,42*). Pb induction of the GRP78 promoter is suggestive of protein structural perturbations (*43*), whereas its induction of the GADD153 promoter suggests a cellular response to DNA damage (*40,44,45*). Both of these responses are suggestive of a cellular response to oxidative damage. Although induction of the XRE, GST Ya, and CYP1A1 promoters is typically suggestive of the presence of aryl hydrocarbons (*35,36*) because there was greater induction of these promoters in the presence of Pb in HA than with HA alone suggests that the induction of these three promoters must be at least partly attributable to Pb. Further work will be needed to elucidate what role Pb may play in inducing expression of these genes. The demonstration of the Pb-induced expression of GST Ya, XRE, CYP1A1, and GADD153 offers new insights into the mechanisms of toxicity and carcinogenicity of Pb and suggests potential new directions for further study on the biologic effects of Pb.

The gene expression profile for Cd in HA in these assays (Figure 6) was similar in several ways to the results seen with As (Figure 4), suggesting possible parallels in the biochemical mechanisms of toxicity for these two metals. The very strong dose-dependent induction of the hMTIIA-regulated reporter by Cd is consistent with previous reports (*26,46,47*). In addition, like As, Cd induced dose-responsive CAT gene expression from the GST Ya and XRE promoter constructs, though the relative levels of induction were different. By contrast with As, however, Cd induced dose-dependent expression of the CYP1A1 promoter–regulated reporter. Induction of the CYP1A1, GST Ya, and XRE cluster of promoters is typically suggestive of the presence of aryl hydrocarbons (*30,35,36,48*). However, as noted previously with As(V), Cd(II), and Pb(II), the magnitude of induction was much greater with Cd in HA than with HA alone (Figure 3), suggesting that Cd must account for much of the observed expression from these promoters. We know of no previous work describing Cd induction of gene expression through the CYP1A1 or XRE promoter. There are, however, earlier reports indicating that Cd exposure increases the concentration of reduced glutathione in various mammalian cell lines (*49,50*) and induces expression of γ-glutamylcysteine synthase (*51*), an enzyme central to the metabolic pathway for glutathione synthesis. Because GST plays a critical role in the conjugation of reduced glutathione with electrophilic xenobiotics (*52*), Cd induction of the GST Ya promoter–regulated reporter may suggest a cellular response to protect against oxidative damage. The coordinate dose-related induction of the NFκBRE–regulated reporter by Cd further suggests an oxidative damage response (*33,34,53*). Cd also produced a moderate dose-dependent induction of the HSP70 promoter, suggesting a cellular response to protein damage (*29,54–56*) and a small dose-related induction of the immediate early proto oncogene promoter *FOS*. Induction of *FOS* is in general agreement with other reports indicating Cd induction of immediate early genes including *FOS*, c-*jun*, and c-*myc* (*49,57–59*) and is suggestive of DNA damage (*60*). As both protein structural perturbations and DNA damage may result from oxidative stress, the induction of the HSP70 and *FOS* promoters may also relate to a cellular response to oxidative stress. These combined results suggest that oxidative stress may comprise an important part of the mechanism of Cd toxicity and carcinogenicity. It is important to note, however, that this may not be a direct effect of the metal, as Cd is not chemically redox active. Finally, the induction of NFκBRE, CYP1A1, XRE, and GST Ya promoters (Figure 6) suggests several signal-transduction pathways for further studies of Cd-mediated effects.

The results observed in testing Cr in these assays present a very interesting picture. The uniform lack of response after treatment with rather high doses up to 500 µM Cr(III) (Figure 7) is consistent with earlier literature indicating little toxicity for Cr(III) and suggesting a role for Cr(III) as an essential trace nutrient [reviewed in Anderson (*61*)]. In contrast, Cr(VI) exposure (Figure 8) produced a profile of gene expression that differed sharply from Cr(III) and other metals used in this study. Differences in the rate of uptake between Cr(VI) and Cr(III) could possibly contribute to these observations. Coordinate dose-related induction of the FOS and NFκBRE promoters observed with Cr(VI) was strongly suggestive of cellular responses to oxidative stress (*32–34,53,60*) and was consistent with earlier reports (*62,63*). At the high dose (10 µM), Cr(VI) induced very strong expression of the p53RE promoter and more moderate expression of the GADD45 and GADD153 promoters. Induction of these three promoters, along with induction of the FOS promoter, suggests a cellular response to DNA damage (*39,40,44,45,60,64*). Induction of the HSP70 and GRP78 promoters, which are both markers of protein structural perturbations, suggests further responses to cellular damage (*29,43,54–56*).

Like As, Cd, and Pb, Cr(VI) induced expression of the CYP1A1, GST Ya, and XRE promoter constructs. Although induction of this set of three promoters is characteristically suggestive of the presence of aryl hydrocarbons (*30,35,36,48*) and may be partly attributable to traces of aryl hydrocarbons in the HA carrier, it is important to note that these three promoters were induced to a much smaller extent by HA alone (Figure 3) and were not induced at all by Cr(III) in HA (Figure 7). Thus, it appears that Cr(VI) directly or indirectly alters the regulation of CAT gene expression by these three promoters. In contrast to As (Figure 4) and Cd (Figure 6), Cr(VI) produced only minimal induction of the metal-responsive hMTIIA promoter. Last, Cr(VI) induced expression of the CRE promoter, suggesting the involvement of the cyclic AMP signal-transduction pathway (*65*). Although these results confirm and extend previous reports of Cr(VI)-mediated expression of oxidative stress genes, they also demonstrate several new signal-transduction pathways, including p53RE, XRE, GADD45, CYP1A1, CRE, GADD153, hMTIIA, and GRP78. These novel pathways offer potential new insights into the mechanisms of Cr toxicity and carcinogenicity.

When a mixture of Cd(II), Cr(III), and Pb(II) was tested in the CAT–Tox (L) assays (Figure 9), hMTIIA was the only promoter strongly induced in a dose-responsive pattern. Induction of the metal-responsive hMTIIA promoter must be primarily attributable to Cd in the mixture, as it was only moderately

induced by Pb alone (Figure 5) and was virtually unaffected by Cr(III) (Figure 7) (*26,27*). The CYP1A1 and XRE promoters were also marginally induced by this mixture, but these results were only significant at the highest dose. Each of these promoters was induced to a smaller extent after treatment with this mixture of metals than by either Cd(II) (Figure 6) or Pb(II) (Figure 5) alone. This finding, along with the observation that these promoters were not appreciably induced by Cr(III) alone (Figure 7), suggests that Cr(III) may actually offer a protective effect by inhibiting induction of these promoters by Cd(II) or Pb(II). In contrast, these results clearly showed no evidence for synergistic activation of gene expression by the three metals in this mixture.

In summary, the results described demonstrate that the four high-priority metals tested in these studies modulate gene expression through signal-transduction pathways not previously associated with these metals. These findings thus suggest new directions for future studies into the biochemical mechanisms of toxicity and carcinogenicity of these metals. Additionally, no evidence was found for synergistic activation of gene expression by a mixture of Cd(II), Cr(III), and Pb(II) tested in this assay.

Results from these types of gene induction studies can be used to address issues related to modes of action, dose–response relationships, chemical interactions, and human exposure assessment (*66*). However, it is important to note that in the cascade of events that occur in a biologic system, the chemically induced alterations of gene expression must lead to qualitative or quantitative changes in the total protein complement, the proteome, of cells and tissues. Such changes in the proteome will likely perturb the homeostasis of an organism. Establishing links between genomics and proteomics is critical for their use in the toxicology and risk assessment of chemicals and their mixtures.

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