Mercury, Cadmium, and Arsenite Enhance Heat Shock Protein Synthesis in Chick Embryos Prior to Embryotoxicity

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BACKGROUND: Cells respond to adverse environmental stimuli by enhancing the expression of specific genes, the products of which include a suite of proteins known as heat shock proteins (hsps), a response often attributed to cellular protection. **METHODS:** In this study, we characterized alterations in hsp expression in chick embryos (Hamburger-Hamilton stage 17, 72 h) exposed in ovo to arsenite (As), mercury (Hg), and cadmium (Cd), known developmental toxicants. Embryos were incubated for 2 h following exposure to 3, 10, 30, or 100 nmol metal, or for 2, 4, 12, or 24 h following treatment with 10 nmol metal. **RESULTS:** An enhanced de novo synthesis of 24-, 70-, and 90-kD, 70- and 90-kD, and 70-kD proteins was observed with As, Hg, and Cd treatments, respectively. These responses were transient; apparent rates of protein synthesis were maximal 2–4 h after exposure and returned to control rates by 24 h. Actinomycin D experiments demonstrated that arsenite-induced expression of these proteins is transcriptionally regulated. Immunoblotting experiments identified the 24-, 70-, and 90-kD proteins as the heat shock proteins hsp24, hsp70, and hsp90, respectively. Exposure duration-related abnormalities were noted in the neural tube with all metals and in the ganglia and somites with Cd and As. Retina, allantois, and limb defects were specific to Cd-treated embryos, and branchial arch defects were specific to As-treated embryos. **CONCLUSIONS:** The data support metal-induced developmental abnormalities, which are preceded by synthesis of stress proteins. *Birth Defects Res B* 68:456–464, 2003. Published 2003 Wiley-Liss, Inc. [†]

Key words: *arsenite; cadmium; mercury; chick embryo; heat shock proteins; teratogenicity; embryotoxicity*

INTRODUCTION

Many cells and organisms have evolved defense mechanisms that protect and repair macromolecules from damage by adverse environmental stimuli. One such strategy is the cellular stress response. A major feature of this response is the rapid expression of a suite of specific proteins referred to as heat shock proteins (hsps). Genes for heat shock proteins encode for constitutive and stress-inducible proteins. In the hsp70 family, levels of the inducible hsp72 increase under conditions of stress, whereas those of the constitutive hsp73 remain fairly unchanged (Kiang and Tsokos, 1998). Hsp expression is highly influenced by exposure to metals, and the specific proteins affected are dependent on metal species, dose, and tissue or organism (Goering and Fisher, 1995; Sanders et al., 1996). Expression of hsp72 in embryos has been shown to increase in response to exposure to several toxic insults including heat (Walsh et al., 1989; Fisher et al., 1995), arsenic (Johnston et al., 1980; Mirkes and Cornel, 1992; Mirkes et al., 1994; Dix, 1997), and cadmium (Andrews et al., 1987). This protein

serves to promote the repair, re-folding, and re-assembly of proteins damaged by chemical and physical stressors (Kiang and Tsokos, 1998). In addition to hsp70, hsp27 and hsp90 synthesis increases in response to toxic agents, such as arsenic (Johnston et al., 1980). Hsp27 is constitutive in most mammalian cells (Dix, 1997) and is important in development, especially during neural plate induction (Walsh et al., 1997). Hsp90 has various cellular functions, including conformational regulation of a

variety of proteins that participate in signaling (Buchner, 1999). In embryogenesis, hsp90 has a role in neural, retina, bone, and muscle development (Csermely et al., 1998).

Several in vitro studies indicate that hsps may protect organisms from metal-induced toxicity during development. Hsp72 expression has been shown to prevent the teratogenic effects of arsenite in the developing mouse (Dix et al., 1998; Hunter and Dix, 2001). Similarly, in mouse embryonic stem cells, overexpression of hsp27 prevented cadmium-, mercury-, and arsenite-induced toxicity (Wu and Welsh, 1996). Earlier reports had indicated that mild heat pre-treatment prevented cadmium-induced teratogenic effects in mouse (Kapron-Bras and Hales, 1992) and rat (Kapron-Bras and Hales, 1991) embryos.

Studies of the potential developmental toxicity of metals in the chick have demonstrated the embryotoxicity of cadmium (Menoud and Schowing, 1987; Gilani and Alibhai, 1990; Thompson and Bannigan, 2001) and mercury (Hughes et al., 1976; Greener and Kochen, 1983). The developmental toxicity of arsenate in the chick has also been examined (Gilani and Alibhai, 1990), but to our knowledge no report exists on the embryotoxic effects of arsenite in the chick. In addition, no study exists on the potential protective effects of hsp27, hsp70, and hsp90 from metal-induced embryotoxicity in an in vivo model. The objective of this study was to expose chick embryos to arsenite, cadmium, and mercury in ovo and examine their teratogenic potential. We studied dose- and time-dependent changes in the de novo synthesis of stress proteins and identified these proteins using immunoblot techniques. We also examined if enhanced synthesis of stress proteins in chick embryos is regulated at the transcriptional level. Finally, the temporal relationship between stress protein synthesis and metal-induced embryotoxicity was compared.

MATERIALS AND METHODS
Chemicals

Sodium arsenite ($NaAsO_2$), mercuric chloride ($HgCl_2$), and actinomycin-D were obtained from Sigma, St. Louis, MO. Cadmium chloride ($CdCl_2$) was obtained from Mallinckrodt, Paris, KY. ^{35}S-methionine (L-^{35}S; 100 μCi/ml; translation grade) was purchased from PerkinElmer Life Sciences, Inc., Boston, MA.

Metal Treatment

Fertile White Leghorn chicken eggs were acquired from Truslow Farms, Inc. (Chestertown, MD) and incubated at 37°C and 65% relative humidity for 72 h to Hamburger and Hamilton (1951) stage 17 before treatment. Chick embryos were treated in ovo by removing shell and shell membranes to form a 1-cm^2 window. A 5-μl aliquot of $NaAsO_2$, $HgCl_2$, or $CdCl_2$ dissolved in 0.9% NaCl or vehicle alone (control) was placed directly on the vasculature surrounding the embryo, and the shell was resealed with Parafilm and replaced in the incubator (Ivnitski et al., 2001). Embryos were exposed to 3, 10, 30, or 100 nmol arsenite, cadmium, and mercury and excised 2 h after exposure, or to saline or 10 nmol metal and harvested 2, 4, 12, or 24 h following

exposure. For actinomycin D experiments, embryos were exposed to 10 μl of a 20 μg/ml solution of actinomycin D in Howard (1953) Ringers solution (HR: 123 mM NaCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl) 30 min prior to a 2-h treatment with 30 nmol arsenite.

Radiolabeling of Tissue Proteins

Following incubation, blastoderms were excised from the yolk, freed of vitelline membranes, and 3 embryos from each treatment group for each of at least 3 separate experiments were pooled in 20-ml glass vials containing 3 ml prewarmed HR solution supplemented with 100 mM glucose, 100 mM sodium pyruvate, and ^{35}S-methionine. Embryos were incubated on a rotator for 60 min at 37°C and then washed three times in Dulbecco's phosphate buffered saline (Invitrogen Life Sciences, Grand Island, NY). Following rinsing, the pooled embryos were transferred to 1.5-ml microcentrifuge tubes and frozen at −70°C for no longer than 5 days.

SDS-PAGE Autoradiography

After thawing on ice, samples were sonicated in 10 mM Tris, pH 7.4, and centrifuged at $15K \times g$ at 4°C for 15 min. Protein in a 5-μl supernatant aliquot was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma, St. Louis, MO) as a standard. Another 5-μl aliquot of the supernatant was added to 10 ml Aquasol-2 cocktail (DuPont, Boston, MA) and radioactivity was measured with a LKB Wallac 1217 Rackbeta Liquid Scintillation Counter. To equalize radiolevels between samples, 20 μl of the sample was diluted with sample buffer (10 mM Tris-HCl, pH 7.4, 2.5% SDS, 2% β-mercaptoethanol, 0.01% bromophenol blue, 1 mM EDTA, pH 8.0). Samples were heated for 5 min at 97°C, chilled on ice, and centrifuged at $15K \times g$ for 5 min.

Proteins in the supernatant were separated on precast 12.5% homogenous acrylamide minigels (Phast System, Pharmacia/LKB Biotechnology, Piscataway, NJ) as described by Goering et al. (1992). Pre-stained molecular weight standards (GIBCO/BRL Life Technologies) were run concurrently. Following electrophoresis, gels were fixed, air dried, and placed on Kodak X-OMAT AR film at −70°C. Images of radiolabeled proteins and pre-stained molecular weight standards were scanned with a laser densitometer (Etruscan 2202, Pharmacia/LKB Biotechnology, Piscataway, NJ) to estimate M_r of sample proteins. Using Gel scan XL software, the area of relevant hsp peaks was calculated and then converted to percent of control. Each data point represents the mean ± standard deviation from at least three different experiments. One-way analysis of variance (ANOVA) was used to assess the variation of the means among the treatments and the Student Newman-Keuls test was performed for a comparison of the means. Significance was established when $P < 0.05$.

Immunologic Detection of Stress Proteins

Aliquots (20 μl) of chick tissue were diluted with sample buffer to equivalent protein concentrations (3 μg/μl). Protein samples and pre-stained molecular weight markers were separated on precast 12.5%

homogenous acrylamide minigels followed by electro-phoretic blotting onto nitrocellulose membranes as described by Goering et al. (1992). The nitrocellulose blots were incubated in Tris-buffered blocking solution containing 4% non-fat dry milk with gentle agitation for 3 h at room temperature. The blots were then washed in TBS and incubated overnight with monoclonal antibodies derived against proteins from the 24-, 70-, and 90-kD heat shock families. A previously characterized rat-anti-chicken hsp70 monoclonal antibody and a rabbit anti-rat hsp24 that cross-reacts with chicken hsp24 (Kelley and Schlesinger, 1982) were a kind gift of Dr. Milton J. Schlesinger, Washington University School of Medicine, St. Louis, MO. The commercially available mouse monoclonal hsp90 antibody (SPA830, StressGen Biotechnologies, Vancouver, BC) has known cross-reactivity with chicken hsp90 (StressGen Biotechnologies). Following incubation with the primary antibody, blots were washed and incubated for 3 h with purified goat anti-rabbit (for detection of hsp24), rabbit anti-rat (for detection of hsp70), or goat anti-mouse (for detection of hsp90) secondary antibodies conjugated with alkaline phosphatase (BioRad Laboratories, Richmond, CA). Detection of the bound antibody complex was performed colorimetrically following incubation with the substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Morphologic Assessment of Embryos

After 72 h of incubation to Hamburger-Hamilton stage 17 (Hamburger and Hamilton, 1951), embryos were treated with 10 nmol metal or 0.9% NaCl (control) as described above, and the eggs were incubated for an additional 4, 12, or 24 h. Each embryo was then excised from the yolk into HR (37°C) and immediately examined for heartbeat as an indicator of survival. The vitelline membrane was removed, and the embryos were fixed in 10% formalin overnight. Further observations of embryonic tissues were conducted in PBS by a single evaluator (KMB). The number of live and dead embryos exhibiting abnormalities in the neural tube, ganglia, retinas, somites, allantois, limbs, and branchial arches was recorded.

RESULTS

De Novo Protein Synthesis

Arsenite, cadmium, and mercury elicited dose-dependent changes in the de novo synthesis of stress proteins (sp) in chick embryos after 2-h exposure to each metal (Fig. 1). Arsenite enhanced the synthesis of three proteins with apparent molecular weights of 24, 70, and 90 kD, denoted as stress proteins sp24, sp70, and sp90, respectively. Treatment of chick embryos with 30 and 100 nmol arsenite significantly increased the de novo synthesis of sp24 (Fig. 1B). Exposure to 10, 30, or 100 nmol arsenite/embryo resulted in an increase in sp70 synthesis to 386, 381, and 360% of control, respectively. A significant increase in sp90 synthesis was observed after treatments with 3, 30, or 100 nmol arsenite/embryo. Cadmium significantly enhanced the de novo synthesis of sp70 when exposed to 3, 10, or 100 nmol/embryo, and mercury significantly increased sp70 synthesis at 30 or 100 nmol/embryo.

The effects of all three metals on the de novo synthesis of sp24, sp70, and sp90 were time-dependent (Fig. 2). Generally, rates were maximal after 2 or 4 h of treatment. Specifically, the maximal effect of arsenite on sp90, sp70, or sp24 synthesis was seen at 4 h (Fig. 2B). Rates returned to baseline at 12 h for sp90 and sp70 and at 24 h for sp24. The effect of cadmium on the de novo synthesis of sp70 was similar after 2 or 4 h of treatment. Cadmium treatment for 12 h significantly lowered the rate of hsp90 de novo synthesis below baseline. Exposure to mercury for 2 or 4 h significantly enhanced synthesis of sp70 and sp90; the rates returned to baseline by 12 h. For the time-course experiments, time-related treatment effects were compared to the 2-h saline control group, since, in a pilot study, no differences in hsp expression were observed in control (treated with saline) embryos after 2, 4, 12, or 24 h of incubation (data not shown).

When chick embryos were treated with actinomycin D 30 min prior to arsenite treatment, the arsenite-induced synthesis of sp24, sp70, and sp90 (Fig. 3, lane 4) was blocked (Fig. 3, lane 5). Treatment with actinomycin D (Fig. 3, lane 3) or vehicle (Fig. 3, lane 2) alone did not enhance expression of these proteins. This result demonstrates that expression of sp24, sp70 or sp90 in chick embryos induced by arsenite is regulated at the level of transcription.

Immunologic Detection of Stress Proteins

The sp24-, sp70-, and sp90-kD proteins were identified as hsp24, hsp70, and hsp90, respectively, using Western immunoblot analysis, and representative blots are shown in Figure 4. The arsenite-induced increase in accumulation of all three proteins was time-dependent. Elevated levels of hsp24 and hsp70 were maximal after 4 h of treatment. Cadmium increased all three proteins; the maximal increases for hsp24, hsp70, and hsp90 were noted after 24, 4, and 2 h of treatment, respectively. Hsp24, hsp70, and hsp90 levels were maximally increased after 24, 4, and 2 h of mercury exposure, respectively.

Embryotoxicity

Exposure in ovo to all three metals resulted in embryotoxicity (Fig. 5). The percentage of abnormal embryos increased with duration of treatment. Of the three metals examined, cadmium was the most embryotoxic (Fig. 5). A 4-h cadmium exposure resulted in 40% abnormal embryos compared to 15% with arsenite and 8% with mercury treatment. Following 12- or 24-h exposure, 100% of the embryos exposed to cadmium were abnormal, compared to 90% with arsenite and 52% with mercury. Embryolethality increased over time with metal treatment. Cadmium was the most embryolethal with lethality rising to 90% with 24 h of treatment. A 4-h arsenite treatment was lethal to only 5% of the embryos, while 12 or 24 h of arsenite treatment were lethal to 45% of the embryos. Mercury treatment did not result in dead embryos with 4 or 12 h of exposure, but embryolethality was 24% after 24 h. Exposing chick embryos to metals produced an array of developmental abnormalities including defects in the neural tube, retina, limbs, cranial ganglia, somites, and branchial arches (Table 1). Cadmium treatment induced abnormalities in the neural tube, ganglia, somites, retina, limbs, and allantois.
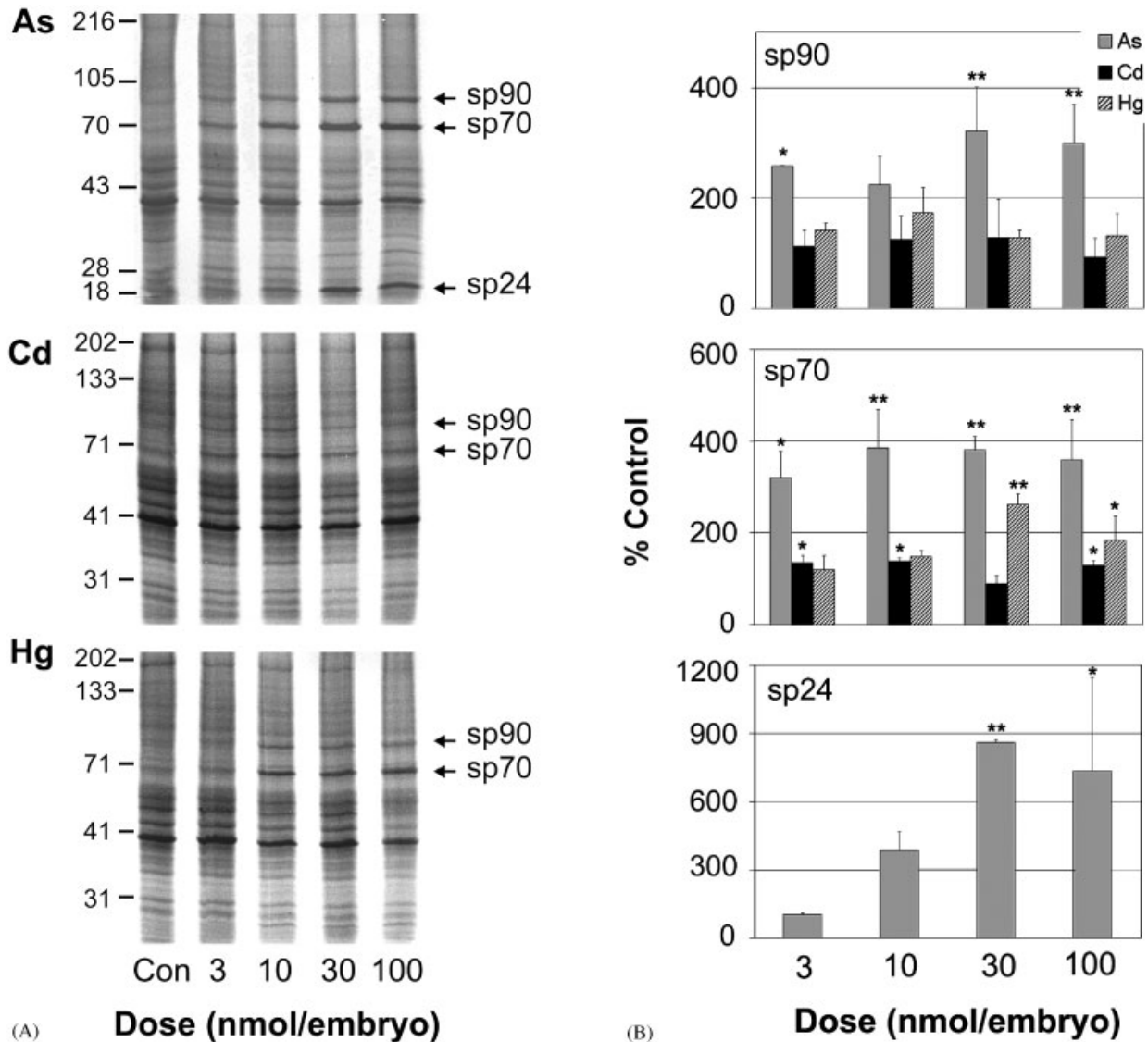
Fig. 1. A: Dose-dependent changes in de novo stress protein synthesis induced in chick embryos exposed to metals. Representative SDS-PAGE (12.5% minigels) profiles of ^{35}S-methionine-labeled chick embryonic proteins 2 h after exposure to 0 (Con), 3, 10, 30, or 100 nmol/embryo of arsenite (As), cadmium (Cd), or mercury (Hg). Each lane was loaded with 120,000 cpm/lane of a supernatant aliquot from a homogenate of 3 pooled embryos. Enhanced de novo synthesis of stress proteins sp90, sp70, and sp24 was observed after As exposure. Cd and Hg induced de novo expression of sp90 and sp70 (see arrows). Molecular weight standards (kilodaltons) are indicated. **B:** Densitometric analysis of dose-dependent changes in de novo synthesis of stress proteins in chick embryos following exposure to arsenite (As), cadmium (Cd), or mercury (Hg). Quantitative changes in de novo synthesis of stress proteins were determined after 2 h exposure to 3, 10, 30, or 100 nmol/embryo of metal by laser densitometric analysis of gel profiles of ^{35}S-methionine-labeled chick embryonic proteins. Density values were calculated as area-under-the-curve and are presented as percent of control. Each data point represents the mean $\pm$ standard deviation from at least three different experiments. [*,**]Significant differences from control as determined by one-way ANOVA and the Student Newman-Keuls test ($P < 0.05$ and $P < 0.01$, respectively).

Arsenite treatment resulted in abnormalities of the neural tube, somites, ganglia, and branchial arches. Mercury treatment produced abnormalities in the neural tube, but no other tissues, after 24 h of treatment.

DISCUSSION

Arsenite, cadmium, and mercury produced rapid, metal-specific qualitative and quantitative differences in hsp expression. Arsenite was the most effective inducer of hsp24, hsp70, and hsp90 de novo synthesis.

Experiments with actinomycin D demonstrated that newly transcribed mRNA is required for the arsenite-induced increases in hsp24, hsp70, and hsp90 levels. In the present study, an increase in the de novo synthesis of hsp24 was first seen with 2 h of treatment, reached a maximum by 4 h, and returned to baseline levels by 24 h. Johnston et al. (1980) observed an arsenite-induced increase in a 27-kD stress protein in chick embryo fibroblasts. In that report, enhanced synthesis of the 27-kD protein was noted at 1 h, reached a maximum by 4 h, which persisted for 1–2 days. In rat embryos, arsenite, but not cadmium, increased hsp72 levels
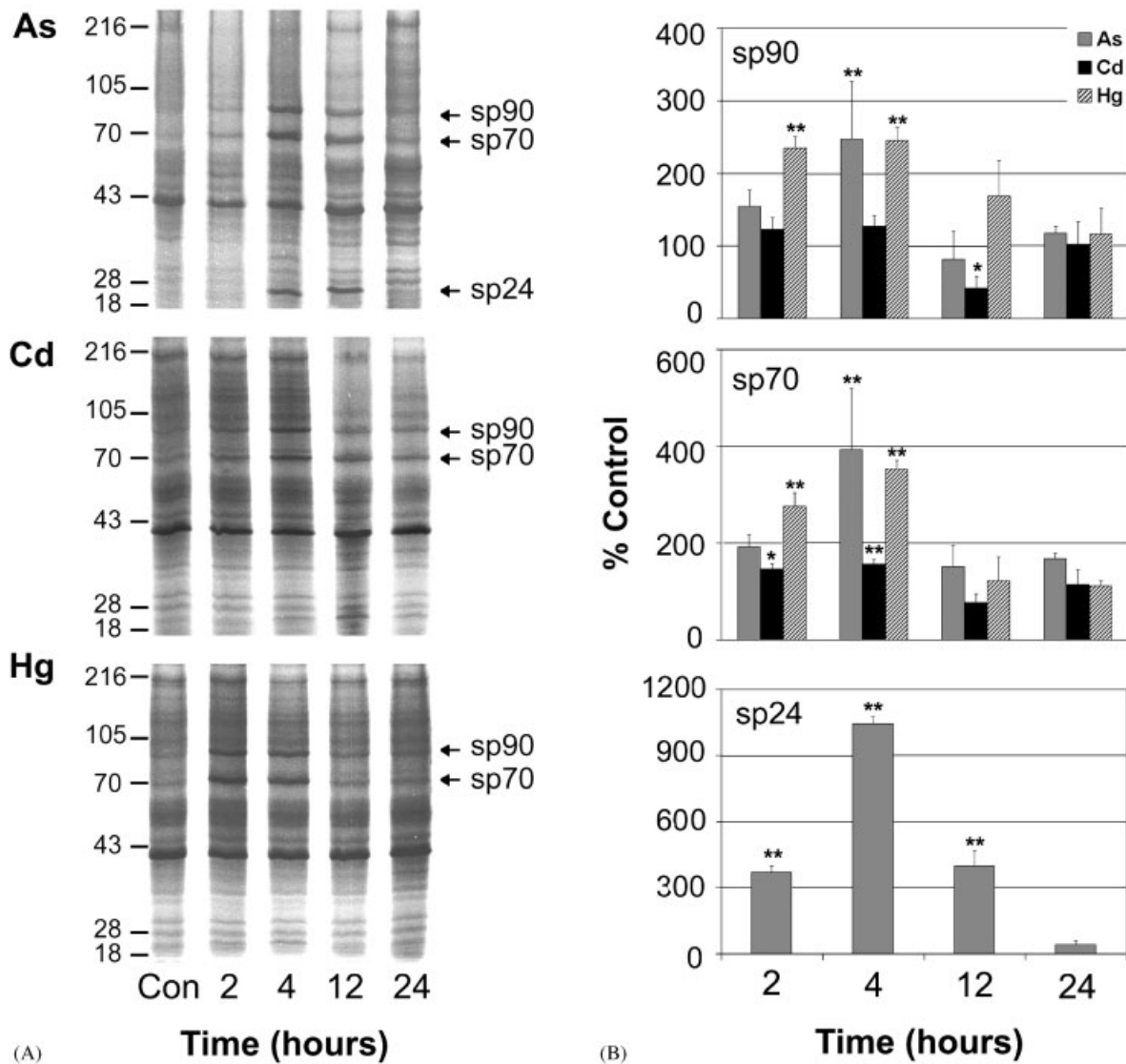
Fig. 2. A: Time-dependent changes in de novo stress protein synthesis induced in chick embryos exposed to metals. Representative SDS-PAGE (12.5% minigels) profiles of ^{35}S-methionine-labeled chick embryonic proteins, 2, 4, 12, and 24 h after exposure to 10 nmol/embryo of arsenite (As), cadmium (Cd), or mercury (Hg). Each lane was loaded with 120,000 cpm/lane of a supernatant aliquot from a homogenate of 3 pooled embryos. Transient enhanced de novo synthesis of stress proteins sp90, sp70, and sp24 proteins was observed after As exposure. Cd and Hg induced transient de novo expression of sp90 and sp70 (see arrows). Molecular weight standards (kilodaltons) are indicated. The control (Con) lane represents proteins separated from embryo homogenates collected after 2 h of incubation; in a pilot study, no differences in hsp expression were observed in control embryos after 2, 4, 12, or 24 h of incubation. **B:** Densitometric analysis of time-dependent changes in de novo synthesis of stress proteins in chick embryos following exposure to arsenite (As), cadmium (Cd), or mercury (Hg). Quantitative changes in de novo synthesis of stress proteins were determined at various times after exposure to 10 nmol metal/embryo by laser densitometric analysis of gel profiles of ^{35}S-methionine-labeled chick proteins. Density values were calculated as area-under-the-curve and are presented as percent of control. Each data point represents the mean $\pm$ standard deviation from at least three different experiments. *,**Significant differences from control as determined by one-way ANOVA and the Student Newman-Keuls test ($P < 0.05$ and $P < 0.01$, respectively).

(Mirkes et al., 1994). An increase in hsp24 content by 24 h in the present study in cadmium- and mercury-treated embryos (Fig. 4) without enhanced de novo synthesis (Fig. 2) suggests that these metals produce post-translational modifications that result in an immunologically detectable protein.

In the present study, cadmium and mercury were the most rapid inducers of hsp70 synthesis. Maximal rates of synthesis were reached after 2–4 h of exposure, which corresponds to other results showing similar maximal rates in rat liver and kidney 2–4 h after injection of

cadmium and mercury, respectively (Goering et al., 1992, 1993). This protein is expressed in mouse embryos beginning at the blastocyst stage and appears to be linked to the development of thermotolerance in heat-treated embryos (Hahnel et al., 1986). Hsp72 accumulates mainly in the neuroectodermal tissue and neural folds of GD10 rat embryos exposed to heat in culture (Fisher et al., 1995) or in utero (Honda et al., 1992). Similarly, in heat-shocked GD9.5 rat whole embryo cultures, hsp72 mRNA accumulation was observed mostly in the neuroectoderm and mesoderm of the neural plate

(Walsh et al., 1989). Cadmium has been shown to increase hsp70 mRNA levels in rabbit blastocysts (Andrews et al., 1987) and hsp70 protein levels in Drosophila embryonic cells (Buzin and Bournias-Vardiabasis, 1984). To our knowledge, the present study is the first to report an enhanced synthesis of hsp70 or hsp90 protein by mercury in a developmental model. This finding is not unexpected since mercury-induced increases in hsp70 mRNA in developing rat embryos have been demonstrated (Li et al., 1998).

All three metals examined were embryotoxic under the exposure conditions of this study. Of the three metals, cadmium was the most embryolethal and induced the most developmental abnormalities. Treatment of chick embryos in ovo with 10 nmol cadmium for 24 h resulted in 10% survival. The main cadmium-induced abnormalities were seen in the neural tube, retinas, allantois, and limbs. Cadmium was the only metal examined that induced abnormal limb and allantois development. The embryotoxicity of cadmium has been studied extensively both in vivo and in vitro in several experimental models. In in vivo studies in the hamster, cadmium treatment resulted in an increased incidence of fetal resorption (Ferm and Carpenter, 1968). Hamster (Ferm and Carpenter, 1968) and rat (Barr, 1973) embryos exposed to cadmium exhibited neural tube defects which included microphthalmia, anophthalmia, encephalocoele, and exencephaly. Similar effects were seen when rat embryos were treated with cadmium in vitro (Zhao et al., 1997). Hamster embryos exposed in vivo to cadmium developed malformed faces (Ferm and Carpenter, 1968). Reported abnormalities induced in vitro by cadmium in the mouse included decreased yolk sac diameter, and head length, somite, neural tube, face and eye defects (Nakashima et al, 1988; Kapron-Bras and Hales, 1991). Treatment of chick embryos in vitro with cadmium resulted in abnormal neural tubes (Kucera and Burnand, 1987; Thompson and Bannigan, 2001), and limbs (Thompson and Bannigan, 2001). Cadmium-induced abnormalities induced in the chick in ovo included eye (Gilani and Alibhai, 1990) and limb defects (Menoud and Schowing, 1987).
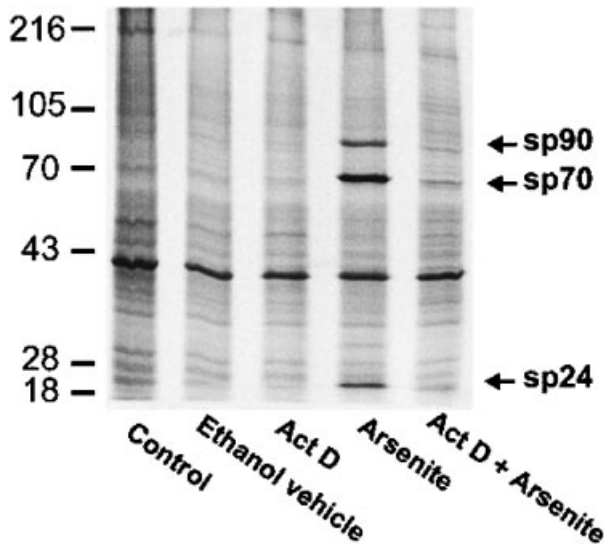

Fig. 3. Effect of actinomycin D (Act D) on stress protein synthesis in chick embryos exposed to arsenite (As). Embryos were pretreated with 10 μl Act D (20 μg/ml) delivered in a Howard Ringers solution 30 min prior to As treatment. Embryos were then exposed to As (30 nmol/embryo) for 2 h and labeled with ^{35}S-methionine for 1 h. Separation of embryonic proteins by SDS-PAGE (12.5% minigels) demonstrated that pretreatment with Act D blocked the enhanced synthesis of the stress proteins sp90, sp70, and sp24 (see arrows). Molecular weight standards (kilodaltons) are indicated.
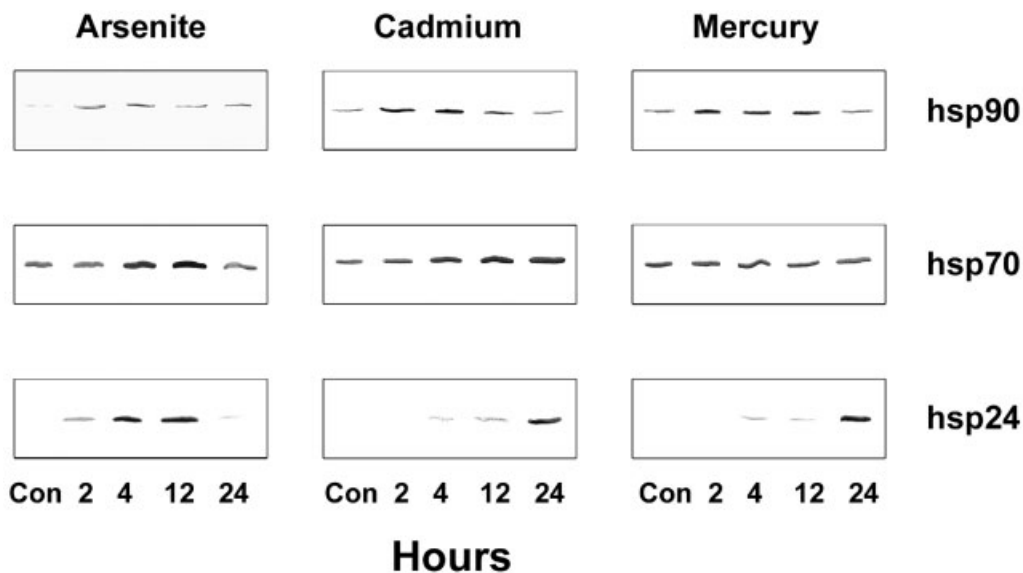

Fig. 4. Immunochemical detection of heat shock proteins in chick embryos at various times after exposure to arsenite (As), cadmium (Cd), or mercury (Hg). After exposure to metal solutions for various times, chick embryonic proteins were separated on 12.5% SDS-PAGE minigels. The 90-, 70-, and 24-kD proteins were identified as hsp90, hsp70, and hsp24, respectively. The control (Con) lane is protein separated from embryo homogenates collected after 2 h of incubation.
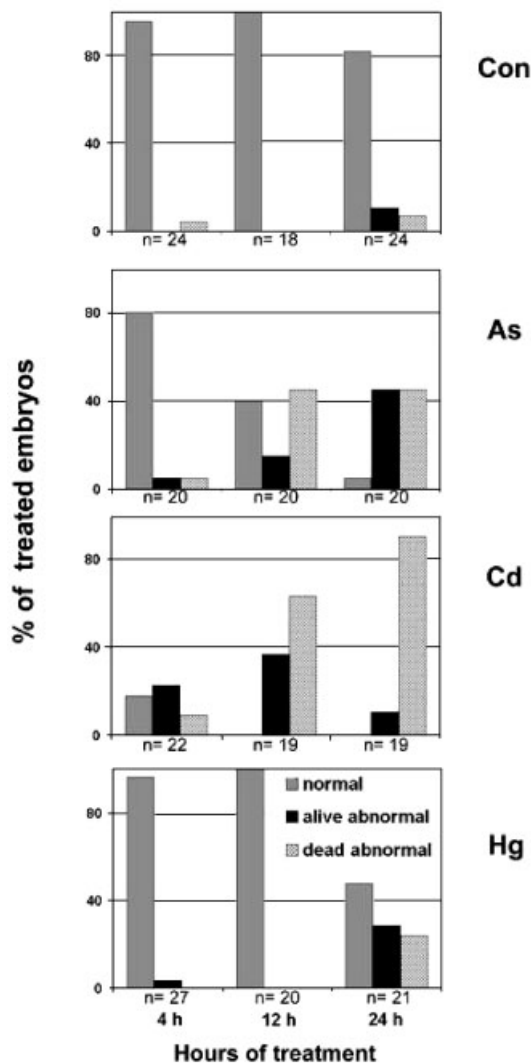
Fig. 5. Effects of metals on chick embryo survival and development. Chick embryos (72 h) were exposed in ovo to 10 nmol/embryo of arsenite (As), cadmium (Cd), or mercury (Hg) and then cultured for an additional 2, 4, 12, or 24 h. Control embryos (Con) were exposed to vehicle only (0.9% NaCl). Survival was determined by a discernable heartbeat. Data were pooled from several experiments conducted on different days (n=19–27 for treated groups and n=18–24 for control groups). Both live and dead embryos at each time point were evaluated for abnormalities (see Table 1).

Arsenite treatment of chick embryos resulted in a time-dependent increase in the number of embryos exhibiting abnormalities. Exposure to arsenite for 12 h resulted in 60% abnormal embryos, while 24 h of exposure resulted in 90% abnormal embryos, half of which were dead. The main deformities of arsenite-treated embryos were in the neural tube, ganglia, somites, and branchial arches. Only one report exists on the potential developmental toxicity of arsenic in the form of arsenate in the chick (Gilani and Alibhai, 1990). In that study, in ovo exposure of chick embryos to arsenate induced lethality and resulted in reduced body and limb lengths and abnormal beaks. Lethality, somite abnormalities, and neural tube defects have been reported as a result of arsenite treatment of

whole mouse embryos in culture (Chaineu et al. 1990; Tabacova et al. 1996).

In the present study, mercury induced death and abnormalities only after 24 h exposure to 10 nmol/embryo. The teratogenic effects of mercury were restricted to the neural tube. In the chick, mercury was shown to decrease the percentage of hatching with the embryos being more sensitive to mercury when exposed in ovo prior to day 7 (Hughes et al., 1976). The mortality rate of mercury-treated chick embryos was dependent on the day of injection, with the mortality being higher in day-5 vs. day-6 chick embryos (Greener and Kochen, 1983).

Metal-induced changes in chick embryo hsp synthesis occurred at earlier exposure durations than those that produce embryotoxicity. For example, whereas arsenite treatment resulted in increases in hsp synthesis as early as 2 h after treatment with maximal induction by 4 h, arsenite-induced abnormalities were first noted 12 h after treatment. Similarly, cadmium increased hsp70 synthesis at 2 and 4, but not at 12 or 24 h after treatment, but its teratogenic potential was mostly observed after 12 and 24 h exposure. Studies in the adult rat have demonstrated that stress protein synthesis precedes arsenite (Wijeweera et al., 1995) and cadmium (Goering et al., 1993) hepatotoxicity.

A possible correlation of hsp expression and the developmental toxicity of metals has been suggested from previous investigations. Overexpression of hsp27 has been shown to protect mouse embryonic stem cells from cadmium-, mercury-, and arsenite-induced lethality (Wu and Welsh, 1996). Increased synthesis of hsp72 and hsp72 mRNA was detected prior to observation of teratogenic effects in rat embryos treated with arsenite in vitro (Mirkes and Cornel, 1992). A correlation between hsp72 accumulation and developmental toxicity in the rat has been established for sodium salicylate, but not for cadmium, cyclophosphamide, or N-acetoxy-2-acetylaminofluorene (Mirkes et al., 1994). Additional developmental toxicants that have been shown to increase synthesis of hsps include heat in the rat (Fisher et al., 1996), phenytoin in the mouse (Hansen et al., 1988), and thalidomide in *Drosophila* cells (Bournias-Vardiabasis and Buzin, 1986). Hsp72 overexpression protects mouse embryos from the teratogenic effects of arsenite (Dix et al., 1998; Hunter and Dix, 2001). Maternal exposure of GD10 mice to retinoic acid results in abnormal forelimbs, hindlimbs, and tail, and accumulation of a 20–25 kD stress protein was seen in all of these tissues shortly after dosing (LaBord et al., 1995). In addition, accumulation of a 90-kD stress protein was observed in the forelimbs and tail, while an 84-kD protein accumulated in the forelimbs. In GD13 mice, maternal exposure to retinoic acid resulted in cleft palate and stress proteins of 34-, 84-, and 90-kD accumulated in craniofacial tissue. In GD11 mice, hindlimbs and forelimbs are the only tissues malformed by retinoic acid treatment, and accumulation of stress proteins of 25-, 84-, and 90-kD were specific to those tissues (Anson et al., 1991). The above studies suggest that stressors increase hsp synthesis in the affected tissues. Fisher et al. (1995), on the other hand, showed that hsp72 was induced by heat in rat neural tissues and not in somites, which were the only structure affected by the heat treatment. The lack of hsp expression in somites may have contributed to their susceptibility to heat. In

TABLE 1
Survival Rates and Abnormalities of Embryos Treated at Hamburger Hamilton Stage 17 (72 h) With 10 nmol of Arsenite (As), Cadmium (Cd), or Mercury (Hg) or 0.9% NaCl Vehicle (Con), and then Incubated for 4, 12, or 24 Hours

Chemical	Hours of treatment	Number of animals	% Survival	Neural tube	Ganglia	Retina	Somites	Allantois	Limb	Branchial arch
Con	4	24	95.8	0	0	0	0	0	0	0
	12	18	100	0	0	0	0	0	0	0
	24	28	92.8	3.6	3.6	0	0	0	0	0
As	4	20	85	5	0	0	0	0	0	5
	12	20	55	25	0	0	0	0	0	0
	24	20	50	30	20	0	25	0	0	25
Cd	4	22	86.4	22.7	0	9.1	0	0	0	0
	12	19	36.0	94.7	21.1	47.4	26.3	36.8	0	0
	24	19	10.5	100	31.6	57.9	26.3	57.9	36.8	0
Hg	4	27	100	3.7	0	0	0	0	0	0
	12	20	100	0	0	0	0	0	0	0
	24	21	76.2	23.8	0	0	0	0	0	0

[a]Specific abnormalities follow. Neural tube: microcephally, open neural tube, crinkled neural tube, incomplete brain segmentation; ganglia: darkened cranial ganglia; retina: reduced retina, optic fissure defects; somites: fused somites, darkened sclerotome; allantois: reduced or absent; limb: reduced limb, abnormal (anterior) projection; branchial arch: darkened mesenchyme.

the present study, specific developmental effects cannot be predicted from hsp levels. Similar hsp developmental profiles induced by different metals may result in different embryonic outcomes due to tissue-specific expression of active hsps. While the current study was not designed to address this specific question, it would be interesting to examine which hsps are present in affected vs. unaffected tissues of metal-treated embryos.

This is the first report to show that hsp24 and hsp90, in addition to hsp70 synthesis, is enhanced by arsenite treatment. We also demonstrate for the first time in an embryo model that mimics in vivo exposures that mercury exposure induces synthesis of hsp70 and hsp90. For all three metals evaluated, hsp expression was shown to precede developmental abnormalities. It is unclear from the present study if in embryos exposed to developmental toxicants, enhanced hsp expression at a certain time prior to the onset of developmental abnormalities confers embryo protection or whether hsps are biomarkers of early tissue injury. The relationship between hsp expression and the potential developmental toxicity of arsenite, cadmium, and mercury merits further investigation.

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