

Neonatal Exposure to Brominated Flame Retardant BDE-47 Reduces Long-Term Potentiation and Postsynaptic Protein Levels in Mouse Hippocampus

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BACKGROUND: Increasing environmental levels of brominated flame retardants raise concern about possible adverse effects, particularly through early developmental exposure.

OBJECTIVE: The objective of this research was to investigate neurodevelopmental mechanisms underlying previously observed behavioral impairments observed after neonatal exposure to polybrominated diphenyl ethers (PBDEs).

METHODS: C57Bl/6 mice received a single oral dose of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) on postnatal day (PND) 10 (i.e., during the brain growth spurt). On PND17–19, effects on synaptic plasticity, levels of postsynaptic proteins involved in long-term potentiation (LTP), and vesicular release mechanisms were studied *ex vivo*. We investigated possible acute *in vitro* effects of BDE-47 on vesicular catecholamine release and intracellular Ca²⁺ in rat pheochromocytoma (PC12) cells.

RESULTS: Field-excitatory postsynaptic potential (f-EPSP) recordings in the hippocampal CA1 area demonstrated reduced LTP after exposure to 6.8 mg (14 µmol)/kg body weight (bw) BDE-47, whereas paired-pulse facilitation was not affected. Western blotting of proteins in the postsynaptic, triton-insoluble fraction of hippocampal tissue revealed a reduction of glutamate receptor subunits NR2B and GluR1 and autophosphorylated-active Ca²⁺/calmodulin-dependent protein kinase II (αCaMKII), whereas other proteins tested appeared unaffected. Amperometric recordings in chromaffin cells from mice exposed to 68 mg (140 µmol)/kg bw BDE-47 did not reveal changes in catecholamine release parameters. Modest effects on vesicular release and intracellular Ca²⁺ in PC12 cells were seen following acute exposure to 20 µM BDE-47. The combined results suggest a postsynaptic mechanism *in vivo*.

CONCLUSION: Early neonatal exposure to a single high dose of BDE-47 causes a reduction of LTP together with changes in postsynaptic proteins involved in synaptic plasticity in the mouse hippocampus.

KEY WORDS: αCaMKII, brain growth spurt, developmental neurotoxicity, field-EPSP recording, hippocampal synaptic plasticity, postsynaptic density. *Environ Health Perspect* 115:865–870 (2007). doi:10.1289/ehp.9860 available via <http://dx.doi.org/> [Online 5 February 2007]

Fetal and neonatal exposure to neurotoxicants have adverse effects on neurodevelopment. Early (small) effects of xenobiotics on the brain could aggravate these effects during development, creating a critical window for neurotoxicity. However, the underlying mechanisms are not well understood (Szipir 2006). Recently, a range of behavioral and neurochemical effects have been described for polychlorinated biphenyls (PCBs) (for review, see Fonnum et al. 2006; Mariussen and Fonnum 2006). Nowadays, the increasing concentrations of the structurally related polybrominated diphenyl ethers (PBDEs) in the environment, human food chain, and human tissues (Hites 2004) raise concern about possible neurotoxic effects. In most samples, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) is the predominant congener. PBDEs are used as flame retardants in a range of products, including electronic equipment, furniture, construction materials, and textiles.

Of concern is that children, at the age of early brain development, accumulate BDE-47

more rapidly than adults because of their diet (breast-feeding/relatively large intake) and behavior (contact with house-dust) (Jones-Otazu et al. 2005). Distribution studies show that developing mice reach higher tissue concentrations of BDE-47 compared with adult mice after identical dosing regimens (Staskal et al. 2006). Behavioral studies have demonstrated adverse neurodevelopmental effects on learning and memory after neonatal BDE-47 exposure. Habituation capability in mice, studied by scoring spontaneous behavior after placement in a new environment, is reduced and this effect is long-lasting and increases with age (Eriksson et al. 2001).

Recently, a proteomics approach was used to investigate the effect of a single oral dose of 12 mg (21.2 µmol)/kg body weight (bw) 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) on brain protein levels in mice, 24 hr after exposure. Levels of striatal proteins associated with neurodegeneration and neuroplasticity and of hippocampal proteins associated with metabolism and energy production

were found to be changed (Alm et al. 2006). It is unclear whether such changes occur after exposure to other congeners, and whether these protein changes have functional consequences.

The main objective of our study was to gain insight in the mechanisms underlying the observed effects of BDE-47 on learning and memory (Eriksson et al. 2001). To this purpose we have investigated *N*-methyl-D-aspartate (NMDA)-dependent long-term potentiation (LTP) in hippocampal slices from animals exposed to a dose of BDE-47 known to induce behavioral aberrations. NMDA-dependent LTP has been used as an electrophysiologic substrate for learning and memories for many years. This form of LTP is induced by tetanic stimulation, strong depolarization, and a large increase in intracellular Ca²⁺ level (for review, see Lynch 2004; Malenka and Nicoll 1999; Soderling and Derkach 2000). Paired pulse facilitation (PPF), a form of short-lasting plasticity that presumably reflects presynaptic function (Xu-Friedman and Regehr 2004), was investigated to reveal possible presynaptic effects of BDE-47. In additional *ex vivo* experiments, we investigated protein expression levels in the postsynaptic density (PSD) and catecholamine release from chromaffin cells to further reveal underlying mechanisms. Acute effects of BDE-47 on intracellular Ca²⁺ and catecholamine release of PC12 cells have been studied *in vitro* to assess the involvement of transient acute effects on potential presynaptic targets. Our findings provide a functional basis for previously observed neurobehavioral changes (Eriksson et al. 2001).

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We thank A. de Groot for excellent technical assistance and L. van Halewijn for hippocampal Nissl staining.

This study was supported by the Faculty of Veterinary Medicine, Utrecht University.

The authors declare they have no competing financial interests.

Received 27 October 2006; accepted 5 February 2007.

Materials and Methods

Animals and chemicals. Male C57Bl/6 mice pups (litters culled to 5 pups each) with mother (Harlan, Horst, the Netherlands) were housed in a standard animal facility on a 12-hr light/dark cycle with food and water *ad libitum*. Animals were treated humanely and with regard for alleviation of suffering. All experimental procedures were performed according to Dutch law and approved by the Ethical Committee for Animal Experimentation of Utrecht University.

Male C57Bl/6 mice received a single oral dose of vehicle [1:10 (wt/wt) mixture of egg lecithin (Sigma-Aldrich, Zwijndrecht, the Netherlands) and peanut oil (*Oleum arachidis*) (Sigma-Aldrich), sonicated with water to obtain a 20% (wt/wt) fat:water emulsion] or 6.8 mg (14 μmol)/kg bw BDE-47 via a metal gastric tube on postnatal day (PND) 10 for field-excitatory postsynaptic potential (f-EPSP) recordings and brain protein analysis, or 68 mg (140 μmol)/kg bw BDE-47 for amperometric recordings of chromaffin cells (to investigate presynaptic effects) at PND17–19. Experimental groups consisted of mice from different nests.

BDE-47 was synthesized and purified (~99%) at the Wallenberg laboratory of Stockholm University. For oral dosing, BDE-47 was dissolved in the egg lecithin/peanut oil mixture and sonicated with water to obtain a 20% (wt/wt) fat:water emulsion.

Hippocampal slice preparation. On PND17–19 (directly after brain growth spurt), the animals were killed by decapitation after inhalation anesthesia (isoflurane), and the brain was rapidly dissected on ice. Hippocampal slices were prepared as described previously (Van der Heide et al. 2005). Briefly, transverse hippocampal slices (450 μm) were cut in ice-cold carbogenated Mg^{2+} -enriched artificial cerebrospinal fluid (ACSF) [containing NaCl (124 mM), KCl (3.3 mM), KH_2PO_4 (1.2 mM), MgSO_4 (2.6 mM), CaCl_2 (2.5 mM), NaHCO_3 (20 mM), and glucose (10 mM)] using a Leica VT1000 S vibratome (Leica Microsystems, Wetzlar, Germany). The slices were allowed to stabilize at room temperature in carbogenated ACSF (MgSO_4 : 1.3 mM) for at least 1.5 hr.

Extracellular recording of field potentials. We recorded f-EPSPs in the CA1 region of hippocampal slices as previously described by Van der Heide et al. (2005), with minor modifications. Slices were superfused with carbogenated ACSF (~2 mL/min) in a recording chamber at 30°C. A bipolar stainless steel stimulation electrode (\varnothing 0.1 mm) was placed on the afferent fibers of the stratum radiatum of the hippocampal CA1 region, as shown in a Nissl-stained hippocampal slice in Figure 1A. f-EPSPs were recorded with ACSF-filled glass microelectrodes using an Axoclamp-2B amplifier

(Axon Instruments, Foster City, CA, USA). Data were digitized and stored using “Spike2” software (Cambridge Electronic Design, Cambridge, UK).

Stimulation intensities for threshold and maximum f-EPSPs were determined. Slices with a maximum response amplitude of ≥ 1 mV were included in the experiment. During baseline recording, half-maximum f-EPSPs were evoked every 30 sec. After 15 min baseline recording, LTP was induced with a single tetanic stimulation (100 Hz, 1 sec) and f-EPSPs were recorded for another 30 min. PPF, with interstimulus intervals of 50, 100, 200, 500, and 1,000 msec, was recorded under identical conditions as for LTP.

For data analysis, we determined initial slopes of the f-EPSPs (Figure 1B). For quantification of LTP, the slope was normalized against the average f-EPSP slope during baseline. Average relative increase of the slope was determined 20–30 min after tetanic stimulation as a measure for LTP and 0–7.5 min after tetanic stimulation as a measure for post-tetanic potentiation (PTP) in the individual animals. To determine PPF, paired-pulse ratio (PPR) was determined by dividing the slope of the second average f-EPSP by the slope of the first average f-EPSP ($n = 10$).

Western blotting analysis. We performed Western blotting analysis as described previously by Gardoni et al. (2006), with minor modifications. The triton-insoluble fraction (TIF) was purified from blind samples of single cortices and hippocampi of control ($n = 4$) and BDE-47-exposed animals [6.8 mg (14 μmol)/kg bw; $n = 4$] using a previously validated biochemical fractionating method (Gardoni et al. 2006), in the presence of protease inhibitors (CompleteTM; Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Similar protein yield was obtained in TIF purified from cortex (~200 μg) and hippocampi (~50 μg) of both groups. Protein composition of this preparation was tested for the absence of presynaptic marker synaptophysin (Gardoni et al. 2001) and enrichment in the PSD proteins (Gardoni et al. 2006). Samples (3 μg) were applied to SDS-PAGE and electroblotted. For each TIF preparation three independent western blotting experiments were run. After blocking nonspecific protein interactions with 10% albumin in Tris-buffered saline (TBS), the nitrocellulose papers were incubated for 2 hr at room temperature with the primary antibodies: NR1 (1:1000; Pharmingen, San Diego, CA, USA), NR2A (1:1000; Zymed, San Francisco, CA, USA), NR2B (1:1000; Zymed), GluR1 (1:1500; Chemicon, Temecula, CA, USA), PSD-95 (1:2000; Affinity BioReagents, Golden, CO, USA), SAP97 (1:1000; StressGen, San Diego, CA, USA), Ca^{2+} /calmodulin-dependent

protein kinase II (αCaMKII ; 1:3000; Chemicon), and p286- αCaMKII (1:1000; Promega, San Luis Obispo, CA, USA) in 3% albumin in TBS. After extensive rinsing in TBS/0.1% Tween 20, the nitrocellulose papers were incubated with horseradish peroxidase-conjugated secondary antibodies. Finally, the antigen-antibody complex was revealed by enhanced chemiluminescence (ECL; Amersham Biosciences, Little Chalfont, UK). Quantification was performed by means of a Quantity-One computer-assisted imaging system (Bio-Rad, Hercules, CA, USA).

Intracellular Ca^{2+} imaging. We investigated acute effects of BDE-47 *in vitro* in PC12 cells. PC12 cells were subcultured in poly-L-lysine-coated glass-bottom cell culture dishes (MatTek, Ashland MA, USA) at 37°C, 5% CO_2 as described previously (Westerink et al. 2000). We used the high-affinity Ca^{2+} -responsive fluorescent dye Fura 2-AM (Molecular Probes; Invitrogen, Breda, the Netherlands) to measure the intracellular Ca^{2+} concentration. PC12 cells were incubated with Fura 2-AM (5 μM , 20 min at room temperature) in saline containing CaCl_2 (1.8 mM), glucose (24 mM), Hepes (10 mM), KCl (5.5 mM), MgCl_2 (0.8 mM), NaCl (125 mM), and sucrose (36.5 mM) at pH 7.3 (adjusted with NaOH). After incubation, the cells were washed with saline and left at room temperature for 15 min to allow intracellular deesterification of Fura 2-AM. After deesterification, the cells were placed on the stage of an Axiovert 35M inverted microscope (Zeiss, Göttingen, Germany) equipped

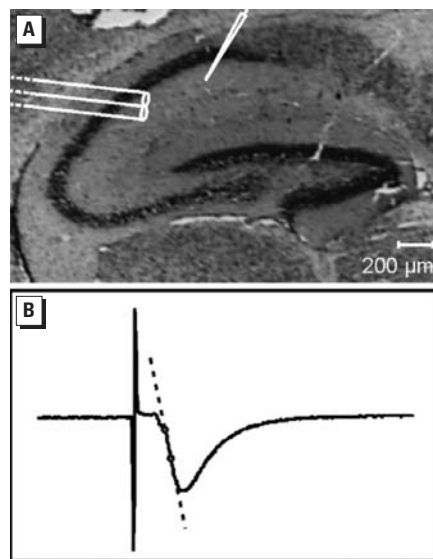


Figure 1. f-EPSP recordings in the CA1 region of hippocampal slices. (A) Placement of bipolar stimulation electrode and ACSF-filled microelectrode in CA1 region in a Nissl-stained hippocampal slice; bar = 200 μm . (B) Representative f-EPSP recording. For data analysis, the initial slope is determined between dots.

with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence evoked by 340 and 380 nm excitation wavelengths (F340 and F380) was collected at 510 nm with an Image SensiCam digital camera (TILL Photonics GmbH). The digital camera and polychromator were controlled by imaging software (TILLvisION, version 4.01), which was also used for data collection and processing. The F340/F380 ratio, which is a qualitative measure for intracellular Ca^{2+} concentration, was measured every 20 sec during baseline. After 5 min baseline recording, BDE-47 was bath-applied to obtain final concentrations of 2 and 20 μM , and ratios were collected every 6 sec. Maximum and minimum ratios were determined after 25 min recording by addition of ionomycin (5 μM) and EDTA (17 mM) as a control for experimental conditions.

Amperometry. We measured spontaneous and K^+ -evoked catecholamine release using carbon fiber microelectrode amperometry from isolated chromaffin cells and PC12 cells as described previously (Westerink and Vijverberg 2002). Chromaffin cells from mice exposed to vehicle or 68 mg (140 μmol)/kg bw BDE-47 were isolated and cultured as described previously (Westerink et al. 2006).

PC12 cells were superfused with BDE-47 for 15 min to investigate acute effects on vesicular catecholamine release. Recordings were performed at room temperature. PC12 cells with high basal release (> 5 events/min) or low evoked release (< 16 events/min) were excluded for data analysis (3/25 cells).

Statistical analysis. All data are presented as mean \pm SE. PC12 data were compared using

Student's paired *t*-test. We first compared the LTP data using a two-way analysis of variance (ANOVA) with *post hoc* Bonferroni testing (Sigmaplot software; Systat Software Inc, Erkrath, Germany), followed by additional unpaired *t*-tests to specify the effects on PTP and LTP. We used unpaired Student's *t*-test for all other data.

Results

Pups exposed to BDE-47 did not differ in body weight and relative thymus weight compared with their unexposed littermates (data not shown), indicating the absence of general toxicity, treatment-dependent food competition, extensive immune suppression, and stress. Additionally, visual inspection of the brain slices of exposed pups did not show any changes of general hippocampus morphology (data not shown).

Figure 2 shows the results from f-EPSP recordings in the CA1 region of mouse hippocampus for control and BDE-47-exposed groups. No differences in stimulus-response relation were seen. No effects were observed on half-maximum f-EPSP slopes before LTP induction (control: 682 ± 138 V/sec; BDE-47 exposed animals: 679 ± 92 V/sec).

After tetanic stimulation, an immediate large increase of the f-EPSP is apparent, although the increase is significantly lower in the BDE-47-exposed group than in the control group. The increase of the f-EPSP during the first 7.5 min post-tetanus is classified as PTP. In the BDE-47-exposed mice, there was significantly less PTP (135 \pm 9%) than in the control mice (190 \pm 17%) ($p < 0.01$) (Figure 2). After PTP the f-EPSP size

decreases but stabilizes at a higher level than baseline. This level of LTP is maintained for at least 30 min. In the BDE-47-exposed mice, LTP was significantly lower (130 \pm 7%) than in the control group (165 \pm 16%) ($p < 0.05$). The significance of these findings was confirmed by two-way ANOVA with *post hoc* Bonferroni testing. The trace inset illustrates the enhancement of f-EPSPs after tetanic stimulation. The cumulative probability curve of LTP in the individual experiments (Figure 2) indicates a shift to lower LTP values in the BDE-47 group.

Figure 3 shows the effect of BDE-47 on PPF at different interstimulus intervals. For the 50-msec interstimulus interval, the PPR was $1.98 \pm 0.11\%$ in the control group and $1.87 \pm 0.15\%$ in the BDE-47 group. For the 1,000-msec interstimulus interval, the PPR was decreased to $1.16 \pm 0.03\%$ in the control group and $1.08 \pm 0.03\%$ in the BDE-47 group. Insets show representative recordings of PPF. No effects of BDE-47 on PPR were detected.

Because activation of NMDA receptors is required for LTP, the reduction of LTP in BDE-47-treated mice could reflect an alteration of NMDA receptor-associated signaling elements. Because the NMDA receptor complex is enriched in the PSD, we used Western blot analysis to measure protein levels of NMDA receptor subunits and other PSD-associated signaling proteins in total homogenate and TIF, representing the PSD compartment by Western blot analysis (Gardoni et al. 2001). Protein composition of this preparation was carefully tested for the absence of presynaptic markers and enrichment in PSD proteins (Figure 4A; Gardoni et al. 2001). Representative Western blots for all investigated proteins in hippocampal homogenate and TIF are also shown in Figure 4B. BDE-47 had no effects on protein levels in cortical homogenate and TIF (data not shown) and hippocampal homogenate

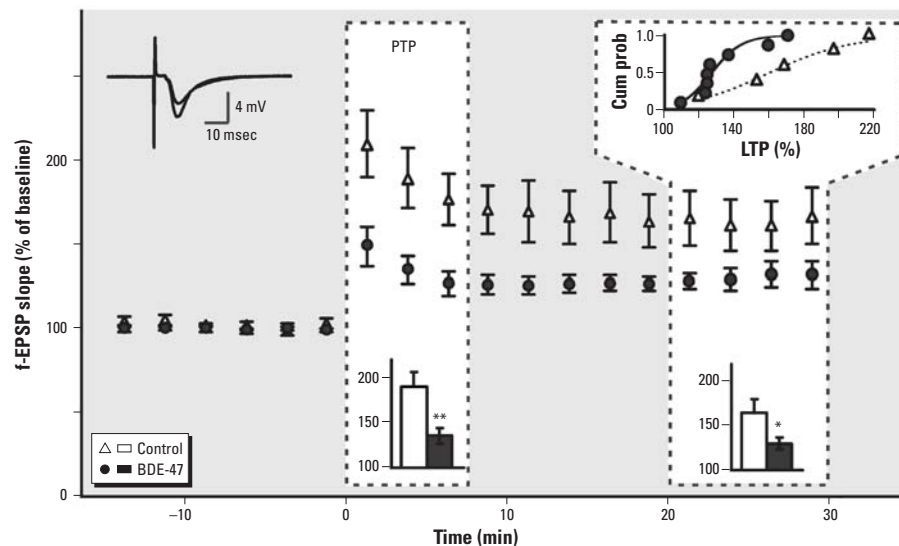


Figure 2. Exposure to 6.8 mg (14 μmol)/kg bw BDE-47 reduces PTP (0–7.5 min after tetanus at $t = 0$) and LTP (20–30 min) in hippocampal neurons in BDE-47-exposed mice ($n = 8$) compared with control mice ($n = 5$). The upper left inset shows superimposed traces illustrating the enhancement of the f-EPSP by LTP induction. The bar diagram insets in the dashed frames show averages of data. The upper right inset shows the cumulative probability (cum prob) curve of LTP in the individual experiments.

* $p < 0.05$; ** $p < 0.01$.

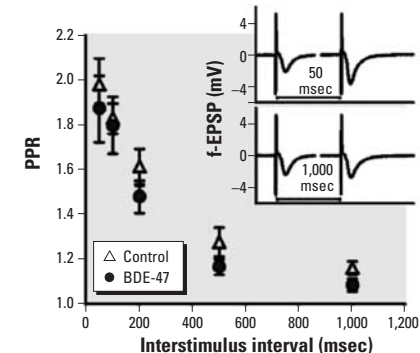


Figure 3. Exposure to 6.8 mg (14 μmol)/kg bw BDE-47 has no effects on PPF; PPR was calculated from paired pulses (interstimulus interval 50–1,000 msec) in control ($n = 12$) and BDE-47-exposed ($n = 12$) mice. Inset shows representative f-EPSP traces of paired pulses.

(Figure 4C). Figure 4C shows amounts of the proteins in TIF of the hippocampus of BDE-47–exposed mice compared with control mice. Significant changes in protein levels of NMDA receptor subunits NR1 and NR2A, and NMDA receptor interacting proteins PSD-95 and SAP97 were not detected. However, protein levels of NMDA receptor subunit NR2B (75 ± 2%) and α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptor subunit GluR1 (71 ± 4%) were significantly reduced ($p < 0.01$). There was a significant decrease in the autophosphorylated-active form of αCaMKII (p286-αCaMKII) to 65 ± 8% of control level ($p < 0.05$), although total αCaMKII was not changed.

In the experiments described above, postsynaptic effects of BDE-47 are observed, whereas presynaptic functional effects are not detected. However, possible effects on presynaptic mechanisms might remain undetected at a dose of 6.8 mg (14 μmol)/kg bw BDE-47. To ascertain the apparent absence of presynaptic effects of BDE-47, we investigated effects on catecholamine release in chromaffin cells obtained from mice exposed to vehicle or to a higher dose (68 mg (140 μmol)/kg bw) of BDE-47. No changes were detected in the different parameters of vesicular catecholamine release; that is, basal and high-K⁺ evoked release frequency and vesicular release parameters like quantal size (vesicle content), spike amplitude, and 50–90% rise time (data not shown).

Additional *in vitro* experiments were performed in PC12 cells to investigate acute effects of BDE-47 exposure on calcium homeostasis and release mechanisms. Figure 5A shows the average F340/F380 ratio in PC12 cells during bath application of DMSO, 2 μM BDE-47, and 20 μM BDE-47 normalized to baseline (first 5 min). The higher concentration of BDE-47 induced an increase in normalized F340/F380 ratio ($t = 12–24$ min, $p < 0.01$). To investigate whether the increase in intracellular Ca²⁺ has functional consequences, vesicular catecholamine release was also investigated (Figure 5B). The average number of

amperometrically recorded events of vesicular release amounted to 1.9 ± 0.7 events/min ($n = 9$) in control experiments. During superfusion with 20 μM BDE-47, the release frequency was enhanced to 6.0 ± 1.7 events/min ($n = 6$; $p < 0.05$), whereas superfusion with 2 μM BDE-47 caused no detectable effect (1.2 ± 0.5 events/min; $n = 7$). BDE-47 had no effect on release evoked by high-K⁺ depolarization of the cells. Differences in vesicular release parameters could not be detected (data not shown).

Discussion

A broad spectrum of neurotoxicants (e.g., environmental pollutants such as metals, pesticides, and PCBs) has been shown to cause a reduction of habituation after neonatal exposure (Eriksson et al. 1990, 1991; Eriksson and Fredriksson 1991; Fredriksson et al. 1992). However, from the behavioral effects it is difficult to deduce information about underlying mechanisms.

In the present study, we found that neonatal exposure to BDE-47 causes developmental effects consisting of a reduction of PTP and LTP, as well as specific reductions of key postsynaptic proteins involved in glutamate receptor signaling. Presynaptic parameters were not affected *ex vivo*. *In vitro* experiments on PC12 cells show an increase in intracellular Ca²⁺ and spontaneous vesicular release, only at the

highest concentration BDE-47 (20 μM). The combined results suggest that presynaptic changes do not directly contribute to the observed defect in synaptic plasticity.

The exposure to BDE-47 took place during a period of rapid brain growth, which in mice takes place during the first 3–4 weeks of life, reaching its peak around PND10 (Davison and Dobbing 1968). The multitude and complexity of processes during this rapid development makes the developing brain particularly vulnerable to the effects of xenobiotics, like the adverse effect of BDE-47 on spontaneous behavior and habituation (Eriksson et al. 2001). Interestingly, exposure to BDE-47 does not affect performance in the Morris water maze test (Eriksson et al. 2001), commonly used as a learning task to detect effects in the hippocampus. This suggests that habituation is a more sensitive parameter for BDE-47 effects in the hippocampus.

We observed a specific reduction of key proteins in the PSD (i.e., GluR1, NR2B, and p286-αCaMKII). Because no changes were observed in total hippocampus homogenate, the specific decrease in the PSD is therefore attributed to changes in glutamate receptor subunit trafficking or clustering in the PSD instead of a reduced protein translation.

A study in GluR1-knockout mice showed that approximately 10% of the normal amount

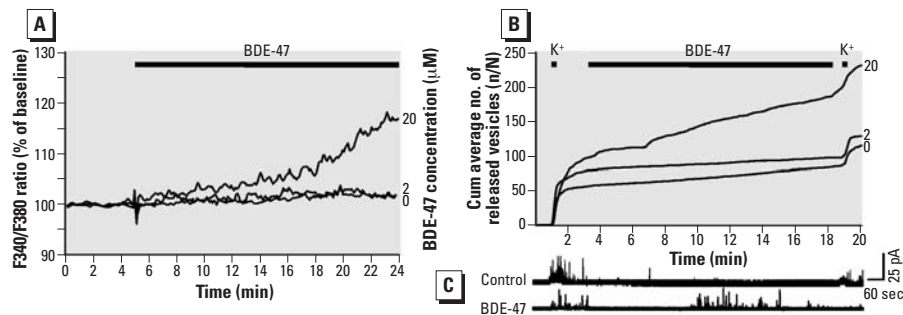


Figure 5. Acute effects of BDE-47 on Ca²⁺ and vesicular catecholamine release in PC12 cells. (A) Intracellular free Ca²⁺ (normalized F340/F380) in cells exposed to DMSO ($n = 79$), 2 μM BDE-47 ($n = 32$), or 20 μM BDE-47 ($n = 27$); base level ($t = 2–4$ min) and effect ($t = 12–14$ min) differed significantly for 20 μM BDE-47. (B) Cumulative (Cum) average number of amperometrically recorded vesicles from PC12 cells exposed to DMSO ($n = 9$), 2 μM BDE-47 ($n = 7$), or 20 μM BDE-47 ($n = 6$). (C) Representative amperometric traces of PC12 cells exposed to DMSO (control) or 20 μM BDE-47.

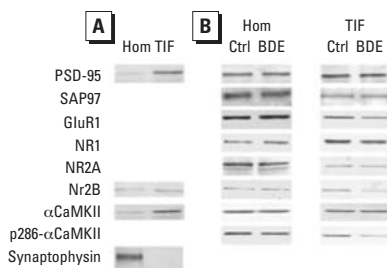
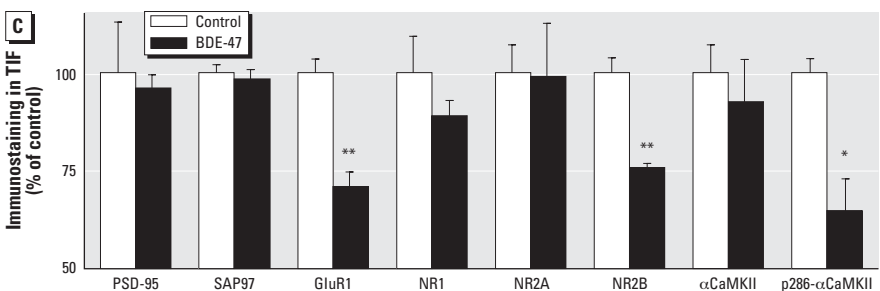


Figure 4. Effects of 6.8 mg (14 μmol)/kg bw BDE-47 on levels of postsynaptic proteins in the hippocampus of control (Ctrl; $n = 4$) and BDE-47–exposed ($n = 4$) mice. (A) Western blotting for NR2B, PSD-95, αCaMKII, and synaptophysin in homogenate (Hom) and TIF from hippocampus. (B) Representative Western blots of the investigated postsynaptic proteins in hippocampal homogenate (Hom) and TIF. (C) Relative amount of postsynaptic proteins in hippocampal TIF (representing the PSD). * $p < 0.05$. ** $p < 0.01$.



of GluR1 is sufficient for LTP (Mack et al. 2001). Also, a GluR1-independent form of LTP has been observed in juvenile GluR1-knockout mice (Jensen et al. 2003). Therefore, major effects on LTP as a consequence of the observed reduction of AMPA subunit GluR1 by approximately 30% are not expected.

The observed reduction of NR2B subunits results in an increased NR2A/NR2B ratio. The majority of NMDA receptors consist of 2 NR1 and 2 NR2A or 2 NR2B subunits. NR2A-NMDA receptors gate smaller Ca^{2+} currents, have a lower affinity for glutamate, and desensitize faster than NR2B-NMDA receptors (Kutsuwada et al. 1992). Therefore, an increased NR2A/NR2B ratio is likely to result in a higher threshold for LTP induction, which could explain the reduction of PTP and LTP.

In mice exposed to BDE-47, the autophosphorylated-active form of αCaMKII was significantly reduced. Because CamKII autophosphorylation is essential for hippocampal NMDA-dependent LTP (Giese et al. 1998), this specific effect may lead to reduced synaptic plasticity resulting in behavioral impairments.

To ascertain the absence of presynaptic effects, we investigated neurotransmitter release from chromaffin cells from BDE-47-exposed [68 mg (140 μmol)/kg bw] mice. Because PPR and chromaffin neurotransmitter release remained unchanged after developmental exposure to BDE-47, and because modest acute effects on free intracellular Ca^{2+} and spontaneous vesicular catecholamine release in PC12 cells were only detected at a concentration of 20 μM BDE-47, we propose that presynaptic changes do not contribute considerably to the observed functional defect in synaptic plasticity. Based on tissue distribution data for 1 mg/kg bw ^{14}C -BDE-47 orally given to C57Bl/6 mice on PND10 (Staskal et al. 2006), brain concentration at sacrifice after exposure to 6.8 mg (14 μmol)/kg bw BDE-47 is estimated to be 0.43–0.81 μM and the peak brain concentration, reached 8 hr after exposure, is estimated to be 1.1 μM . These estimated concentrations are at least one order of magnitude lower than the lowest effective concentration in the *in vitro* experiments described here.

As with PCBs (for review, see Fonnum et al. 2006), *in vitro* exposure to the commercial penta-BDE mixture DE-71, which contains (on a weight basis) 31.8% BDE-47 (Reistad and Mariussen 2005), affects several other transmitter systems. Previous studies reported cell death of cerebellar granule cells, alterations of Ca^{2+} homeostasis in human neutrophils and brain microsomes, and arachidonic acid release and protein kinase C translocation in cerebellar granule cells; inhibition of dopamine reuptake in rat brain synaptic vesicles has been reported after

in vitro exposure to DE-71 in the micromolar range (2–20 μM) (Kodavanti and Ward 2005; Mariussen and Fonnum 2003; Reistad et al. 2002; Reistad and Mariussen 2005). Interestingly, addition of the NMDA receptor antagonist MK801 protects cerebellar granule cells against DE-71-induced cell death (Reistad et al. 2006). No other effects of PBDEs on glutamate receptors have yet been published.

Pure (~99%) BDE-47, which has been used in only a few experiments, has revealed formation of reactive oxygen species in human neutrophils and increased ^3H -phorbol ester binding in primary rat cerebellar granule neurons, also at micromolar concentrations (Kodavanti et al. 2005; Reistad and Mariussen 2005). The effects of BDE-47 in PC12 cells reported here occur at concentrations in the same order of magnitude.

Effects on spontaneous motor activity and habituation in mice have been described for several lower and higher brominated diphenyl ethers after a single oral dose of maximally 21 μmol /kg bw (Branchi et al. 2002, 2003; Eriksson et al. 2001, 2002; Viberg et al. 2003a, 2003b, 2006). In rats, effects on behavior have been observed after maternal exposure to 10 mg (18 μmol)/kg bw BDE-99 at gestational days 10–18 and after oral exposure to 30 mg/kg bw DE-71 at PND6–12 (Dufault et al. 2005; Lilienthal et al. 2006).

In the 1990s, an association between delayed human neurodevelopment and prenatal or early exposure to PCBs was reported by cohort studies. These results were corroborated by experiments demonstrating the developmental neurotoxicity of PCBs. The observed interaction with the thyroid hormone system is usually considered part of the underlying mechanism (for review, see Winneke et al. 2002). For hazard characterization of PCBs and the structurally related PBDEs, it is relevant to investigate whether they induce similar effects through similar mechanisms. This is of particular importance because, in neonatal mice, the effects of a combined dose of PCB-52 and BDE-99 on spontaneous motor behavior and habituation capability appear to be additive or perhaps even synergistic (Eriksson et al. 2006).

High human serum concentrations of BDE-47 were measured in female inhabitants of California by Petreas et al. (2003); the concentration of BDE-47 in serum ranged from 5 to 510 ng/g lipid weight, with a median of 16.5 ng/g lipid weight. High concentrations (> 100 ng/g lipid weight) have also been reported in Californian children (Fisher et al. 2006). The highest and median values correspond (using average physiologic values) to blood concentrations of approximately 11.5 nM and approximately 0.37 nM. Using the tissue distribution data for 1 mg/kg bw ^{14}C -BDE-47 (Staskal et al. 2006), the dose

used in the current study corresponds to an estimated blood concentration of approximately 2.6 μM after 3 hr and to approximately 0.6 μM after 10 days (i.e., ~50–200 times higher than in the worst, and ~1,600–7,000 times higher than in the median human situation described above). For risk assessment, the difference between the animal dose level causing an adverse effect and the highest human dose levels is relatively small, considering safety factors for species extrapolation and intraspecies variability. Additional uncertainty comes from the fact that humans are exposed to multiple flame retardants over a lifetime. Accumulation of BDE-47, as demonstrated in primary rat cerebellar granule neurons and primary rat neocortical cells (Kodavanti et al. 2005; Mundy et al. 2004), is another reason for concern about the neurotoxic potential of PBDEs.

No tolerable daily intake is assigned to PBDEs because sufficient data are not available. However, the limited toxicity data suggest that adverse effects induced by exposure to the more toxic congeners in rodents occur at doses of at least 100 μg /kg bw per day [Joint FAO/WHO Expert Committee on Food Additives (JECFA) 2005]. The combination of quantitative molecular data with functional neurophysiologic effects reported here provides strong functional support for the previously reported neurobehavioral effects (Eriksson et al. 2001) and is essential for characterization of the neurotoxic hazard of brominated flame retardants, particularly for rational risk assessment, which is required in response to the general concern about the vulnerability of the developing brain.

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Lead Levels and Ischemic Heart Disease in a Prospective Study of Middle-Aged and Elderly Men: the VA Normative Aging Study

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BACKGROUND: Lead exposure has been associated with higher blood pressure, hypertension, electrocardiogram abnormalities, and increased mortality from circulatory causes.

OBJECTIVE: We assessed the association between bone lead—a more accurate biomarker of chronic lead exposure than blood lead—and risk for future ischemic heart disease (IHD).

METHODS: In a prospective cohort study (VA Normative Aging Study), 837 men who underwent blood or bone lead measurements at baseline were followed-up for an ischemic heart disease event between 1 September 1991 and 31 December 2001. IHD was defined as either a diagnosis of myocardial infarction or angina pectoris that was confirmed by a cardiologist. Events of fatal myocardial infarction were assessed from death certificates.

RESULTS: An IHD event occurred in 83 cases (70 nonfatal and 13 fatal). The mean blood, tibia, and patella lead levels were higher in IHD cases than in noncases. In multivariate Cox-proportional hazards models, one standard deviation increase in blood lead level was associated with a 1.27 (95% confidence interval, 1.01–1.59) fold greater risk for ischemic heart disease. Similarly, a one standard deviation increase in patella and tibia lead levels was associated with greater risk for IHD (hazard ratio for patella lead = 1.29; 95% confidence interval, 1.02–1.62).

CONCLUSIONS: Men with increased blood and bone lead levels were at increased risk for future IHD. Although the pathogenesis of IHD is multifactorial, lead exposure may be one of the risk factors.

KEY WORDS: angina, epidemiology, myocardial infarction. *Environ Health Perspect* 115:871–875 (2007). doi:10.1289/ehp.9629 available via <http://dx.doi.org/> [Online 6 February 2007]

Although blood lead levels in the United States and other industrialized nations have declined over the past decades, pockets of high lead exposure and widespread low-level lead exposures still persist (Pirkle et al. 1994). Moreover, a substantial proportion of the population has had higher lead exposure from leaded gasoline and other sources such as soldered cans, paints, and tap water in the past (Pirkle et al. 1994). The long-term consequences of lead exposure include circulatory diseases, kidney diseases, and neurologic disorders (Cheng et al. 2001; Harlan 1988; Hertz-Picciotto and Croft 1993; Hu et al. 1996a; Kopp et al. 1988; Lin et al. 2003; Martin et al. 2006; Moller and Kristensen 1992; Nash et al. 2003; Pirkle et al. 1985; Schwartz 1991, 1995; Tsaih et al. 2004).

Lead exposure has been associated with increased blood pressure and hypertension in cross-sectional as well as longitudinal studies (Cheng et al. 2001; Harlan 1988; Hertz-Picciotto and Croft 1993; Hu et al. 1996a; Kopp et al. 1988; Martin et al. 2006; Moller and Kristensen 1992; Nash et al. 2003; Pirkle et al. 1985; Schwartz 1991, 1995). More recently, there is evidence of increased mortality from circulatory causes in individuals with blood lead levels of 20–29 µg/dL in the past (Lustberg and Silbergeld 2002). However, the association between lead levels

and risk for future ischemic heart disease (IHD) after controlling for potential confounders has not been established. The three previous reports on the possible association between lead levels and cardiovascular disease found no such evidence (Kromhout 1988; Moller and Kristensen 1992; Pocock et al. 1988). These reports used blood lead level as a biomarker for lead exposure, which is now known to poorly reflect the cumulative internal dose of lead. Instead, more recently, bone lead has become the biologic marker of choice to assess long-term lead exposure (Landrigan 1991). With the development of *in vivo* K X-ray fluorescence (KXRF), it is now possible to safely and rapidly measure bone lead in large-scale epidemiologic studies (Landrigan and Todd 1994).

The objective of our study was to assess the relationship of bone and blood lead levels with risk for IHD (fatal and nonfatal) in a longitudinal cohort of aging men.

Materials and Methods

Study population. Participants in our study were from the Normative Aging Study (NAS), a longitudinal study of aging established by the Veterans Administration (now Department of Veterans Affairs) in 1961 (Bell et al. 1972). The study cohort initially consisted of 2,280 community-dwelling men who

were health-screened from the Greater Boston area; those with chronic medical conditions such as heart disease, diabetes, cancer, peptic ulcer, gout, recurrent asthma, bronchitis, or sinusitis were excluded. Those with either systolic blood pressure > 140 mm Hg or diastolic blood pressure > 90 mm Hg were also excluded. The men were between 21 and 80 years of age (mean, 42 years) on entry into the cohort. Participants subsequently returned for examinations every 3–5 years during the follow-up period. At each visit, extensive physical examination, laboratory, anthropometric, and questionnaire data were collected.

Measurement of blood lead began in 1988 during each continuing regularly scheduled visit of the participant. Beginning in September 1991, permission was sought from

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We gratefully acknowledge the assistance of K. Croom in managing the Normative Aging Study databases and programming the extraction of dataset for analysis. We are also, as always, indebted to the continued enthusiastic cooperation of participants in the VA Normative Aging Study.

Support for this research was provided by National Institute of Environmental Health Sciences (NIEHS) grants ES 05257 and P42-ES05947 (with funding from the U.S. Environmental Protection Agency), National Institutes of Health (NIH)/National Center on Minority Health and Health Disparities P20 MD000501, and NIEHS Occupational and Environmental Health Center grant no. 2 P30 ES00002. The Normative Aging Study is supported by the Co-operative Studies Program/Epidemiology Research and Information Centers (ERIC) of the U.S. Department of Veterans Affairs and is a component of the Massachusetts Veterans Epidemiology Research and Information Center (MAVERIC), Boston, MA. Subjects were evaluated in the outpatient Clinical Research Center of the Brigham and Women's Hospital with support from NIH grant NCRR GCRC M01RR02635. The KXRF instrument used in this work was developed by ABIOMED, Inc., of Danvers, Massachusetts, with support from NIH grant SBIR 2R44 ES03918-02.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry.

The authors declare they have no competing financial interests.

Received 18 August 2006; accepted 6 February 2007.

each participant to obtain KXRF bone lead measurements. Consenting individuals reported to the Ambulatory Clinical Research Center of the Brigham and Women's Hospital in Boston. Of the 1,278 participants seen for their regularly scheduled NAS visits from 1 September 1991 through 31 December 2001, our study included participants who had information on either blood or bone lead level and had at least one follow-up visit in this time frame ($n = 1,019$). The major reason given for nonparticipation in the bone lead study was the inconvenience involved in making a separate visit to our bone lead test facility. After excluding participants with a history of IHD (myocardial infarction or angina) before their year of baseline lead measurement visit, the final data set for analysis included 837 participants. These 837 participants had their baseline lead measurement done during their first scheduled visit after September 1991. Approval for this study was obtained from the Human Research Committees of Brigham and Women's Hospital and the Department of Veterans Affairs Outpatient Clinic. This study complied with all applicable requirements of the United States (including institutional review board approval), and all participants gave written informed consent before the study.

History and physical parameters. Each NAS participant reported to the study center in the morning after an overnight fast and abstinence from smoking. At the start of the visit, height and weight were measured with the participant wearing only stockings and undershorts. A complete medical history, including identity and purpose of medications taken daily, was elicited by a physician. A history of physician-diagnosed diabetes mellitus and hypertension since the last visit was also elicited. A participant was considered as having a family history of hypertension if either a parent or a sibling had hypertension. The American Thoracic Society questionnaire (Ferris 1978) was used to assess current smoking and past history of smoking, and the Food Frequency Questionnaire (Ward et al. 1994; Willett et al. 1988) was used to assess alcohol consumption.

The participants were asked about history of heart disease since their last visit. Every report of IHD event was reviewed by a board-certified cardiologist, who was unaware of the participant's blood and bone lead levels. The criteria for myocardial infarction and angina pectoris were those used in the Framingham Heart Study (Shurtleff 1974). A diagnosis of myocardial infarction was defined by unequivocal electrocardiographic changes (i.e., pathologic Q waves), diagnostic increases in serum glutamic-oxaloacetic transaminase and lactic dehydrogenase, and concurrent chest discomfort consistent with

myocardial infarction, or by autopsy. Angina pectoris was diagnosed when the participant reported recurrent chest discomfort that lasted up to 15 min and was distinctly related to exertion and relieved by rest or nitroglycerin. Events of fatal IHD were assessed from death certificates. Regular mailings to NAS participants were used to maintain vital status information, and death certificates were obtained for all decedents.

Immediately after the history was obtained, blood pressure was measured using a standard mercury sphygmomanometer with a 14-cm cuff by a physician. With the subject seated for at least 3 min, systolic blood pressure and fifth-phase diastolic blood pressure were measured in each arm to the nearest 2 mm Hg. The means of the right and left arm measurements were used as each participant's systolic and diastolic blood pressures.

Blood lead measurements. Blood samples for lead measurement were taken in special trace-metal-free tubes containing ethylenediaminetetra-acetic acid, and sent to ESA Laboratories, Inc. (Bedford, MA), for analysis. After room temperature digestion with nitric acid, the sample solution was centrifuged and the supernatant was poured into a sample cup. It was then analyzed by Zeeman background-correlated flameless atomic absorption (graphite furnace). The instrument was calibrated after every 21 samples with National Bureau of Standard Blood Lead Standards materials (Gaithersburg, MD). Ten percent of the samples were run in duplicate; at least 10% of the analyses were controls and 10% were blanks. A complete calibration check was made after the last specimen was analyzed. In tests on reference samples from the Centers for Disease Control and Prevention (Atlanta, GA), the coefficient of variation ranged from 8% for concentrations < 10 to 30 $\mu\text{g}/\text{dL}$, to 1% for higher concentrations. In comparison to a National Bureau of Standards (Gaithersburg, MD) target with a known blood lead concentration of 5.7 $\mu\text{g}/\text{dL}$, 24 repeated measurements conducted by ESA Labs using this method gave a mean \pm SD of 5.3 ± 1.23 $\mu\text{g}/\text{dL}$.

KXRF bone lead measurements. Bone lead measurements were performed from each participant's mid-tibial shaft and patella with a KXRF instrument (ABIOMED Inc, Danvers, MA). The physical principles, technical specifications, validation, and quality control procedures of this (Burger et al. 1990; Hu et al. 1990, 1994) and other KXRF instruments (Jones et al. 1987; Somerville et al. 1985) are described elsewhere. Because this instrument provides a continuous unbiased point estimate that oscillates around the true bone lead value, negative point estimates are sometimes produced when the true bone lead value is close to zero. An estimate of the uncertainty associated

with each instrument, derived from a goodness-of-fit calculation of the spectrum curves and equivalent to a single standard deviation, is also provided. Although a minimum detectable limit calculation of twice this value has been proposed for interpreting an individual's bone lead estimate (Gordon et al. 1993), retention of all point estimates makes better use of the data in epidemiologic studies (Kim et al. 1995). As a standard quality-control procedure of KXRF measurements, tibia and patella bone lead measurements with uncertainty estimates of > 10 $\mu\text{g}/\text{g}$ and > 15 $\mu\text{g}/\text{g}$, respectively, of bone mineral were excluded. For our study, 30-min measurements were taken at the mid-shaft of the left tibia (representing cortical bone) and at the left patella (representing trabecular bone), after each region was washed with a 50% solution of isopropyl alcohol. The KXRF beam collimator was sited perpendicular to the bone surface for the tibia and 30 degrees in the lateral direction for the patella.

Statistical analysis. We calculated univariate statistics and examined them for cases and noncases of IHD. We used chi-square or *t*-tests to assess the difference across cases and noncases. Blood and bone lead levels were log-transformed because their distributions were skewed. A value of 35 was added to tibia and patella lead levels before log-transformation (Kim et al. 1995; Kosnett et al. 1994).

We assessed the association between lead levels and risk for subsequent development of new IHD using Cox's proportional hazards models. The follow-up period started at the time of baseline visit (after 1 September 1991) and lasted until the time of first IHD event or death from myocardial infarction, whichever occurred first. If the participant did not have an IHD event, the follow-up period ended on the date of last visit (before 31 December 2001) or 31 December 2001 (if the participant had a visit after 31 December 2001). Because only the year of IHD event was available, 31 December of the year in which the event occurred was used to calculate person-years for all incident cases.

We selected possible confounders on the basis of their biologic significance and information from previous studies. These covariates included age, body mass index, education, race, current smoking status, pack-years smoked, alcohol intake (grams per day) (Moller and Kristensen 1992; Pocock et al. 1988), history of diabetes mellitus and hypertension (Barzilay et al. 1998; Castelli et al. 1989), family history of hypertension, diastolic and systolic blood pressure, serum triglycerides, serum high-density lipids, and total serum cholesterol. Variables significant at the 0.10 level in univariate models were included in final multivariate models. Each of the log-transformed lead biomarker variables (blood lead, tibia lead, and patella lead) was

then added separately into the multivariate models. We also analyzed blood lead as a categorical variable (≥ 5 $\mu\text{g}/\text{dL}$, ≥ 10 $\mu\text{g}/\text{dL}$, and ≥ 15 $\mu\text{g}/\text{dL}$) and bone lead in tertiles. To check for any residual or negative confounding, all covariates were again added, one at a time, in the final regression models. We performed a sensitivity analysis for all final regression models after excluding patients with diabetes mellitus (Barzilay et al. 1998; Castelli et al. 1989).

Statistical analysis was performed using SAS for UNIX (version 9.0; SAS Institute Inc., Cary, NC). The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

A comparison of participants included in our study with nonparticipants in the KXRF bone lead study, within the same time frame, revealed no significant differences with respect to age, race, body mass index, alcohol intake, smoking, a family history of hypertension, systolic and diastolic blood pressure, and a history of diabetes mellitus or hypertension (data not shown). A similar comparison of participants included in our study with those who did not return for a follow-up visit during our study time frame also yielded no significant differences between the two groups.

Of the 837 participants in our study, an IHD event occurred in 83 cases (70 nonfatal and 13 fatal). The mean age of noncases (65.9 \pm 7.3 years) was similar to that of cases (67.5 \pm 6.5 years). The distribution of other covariates—including known risk factors for IHD such as smoking, alcohol intake, systolic and diastolic blood pressures, a history of diabetes mellitus or hypertension, serum triglycerides, and total serum cholesterol—was also similar among cases and noncases (Table 1). However, the person-time contributed by noncases was significantly longer than cases, because cases were censored once an IHD event occurred.

The mean blood, tibia, and patella lead levels were higher in IHD cases than in noncases. When blood lead level was examined as a categorical variable, the proportion of cases with a blood lead level ≥ 5 $\mu\text{g}/\text{dL}$ was significantly higher than noncases. When bone lead was examined in tertiles, a higher proportion of cases were in the highest tertile of tibia and patella lead level compared with noncases (for tibia lead: 38.1% cases compared with 32.7% noncases; for patella lead: 49.2% cases compared with 30.8% noncases) (data not shown).

Age and serum high-density lipids were associated with IHD in multivariate Cox proportional hazards regression models such that the risk for IHD increased with increasing age and decreased with increasing serum high-density lipids (Table 2). When assessed as continuous variables, an increase in blood or

bone lead level was associated with higher risk for an IHD event. As a categorical variable, blood lead level ≥ 5 $\mu\text{g}/\text{dL}$ had a hazard ratio of 1.73 [95% confidence interval (CI), 1.05–2.87] for IHD compared with blood lead level < 5 $\mu\text{g}/\text{dL}$. A dose response was not noted when tibia and patella lead levels were analyzed in tertiles and quartiles.

The inclusion of other covariates known to be risk factors for coronary disease—such as body mass index, alcohol consumption, current smoking, pack-years, a diagnosis of diabetes, a diagnosis of hypertension, blood pressure, family history of hypertension, total serum cholesterol, and total serum triglycerides—in the final regression models did not alter our findings on the association between lead and IHD (data not shown). Our results were also similar when participants with diabetes were excluded from the analysis (data not shown).

The correlation between blood and bone lead levels was modest (correlation coefficient = 0.30 for tibia and blood lead, and 0.37 for patella and blood lead). As expected, tibia and patella lead levels were strongly correlated with each other (correlation coefficient = 0.78). When blood lead and one of the bone

lead variables were assessed in regression models simultaneously, the individual effect estimates of blood and bone lead were only moderately attenuated. The hazard ratio for log blood lead was 1.24 (95% CI, 0.80–1.93) and for log patella lead was 2.62 (95% CI, 0.99–6.93) when these variables were assessed together in a multivariate model. Similarly, the hazards ratio for blood lead was 1.38 (95% CI, 0.89–2.13) and that for tibia lead was 1.55 (95% CI, 0.44–5.53) when these variables were included together in the model.

Discussion

The relationship between biomarkers of long-term lead exposure and IHD has not been previously assessed. In a longitudinal study of 837 middle-aged and elderly men followed from 1 September 1991 through 31 December 2001, we found that the risk of future IHD increases significantly with increasing bone and blood lead levels, after adjusting for potential confounders.

The relationship of lead exposure with hypertension and increased blood pressure has been established in previous studies (Cheng et al. 2001; Harlan 1988; Hertz-Picciotto and Croft 1993; Hu et al. 1996a; Kopp et al. 1988;

Table 1. Baseline characteristics of IHD cases and noncases, Normative Aging Study, 1991–2001.

Characteristic	Noncases (n = 754)			Cases (nonfatal n = 70; fatal n = 13)		
	Total ^a	No. (%)	Range	Total ^a	No. (%)	Range
Age (years)*						
< 60	754	162 (21.5)	—	83	10 (12.1)	—
60–69	754	378 (50.1)	—	83	48 (57.8)	—
≥ 70	754	214 (28.4)	—	83	25 (30.1)	—
Race**						
White	747	734 (98.3)	—	82	78 (95.1)	—
Black	747	13 (1.7)	—	82	4 (4.9)	—
Current smoker	754	60 (8.0)	—	83	4 (4.8)	—
Pack-years (among smokers) ^b	528	29.7 \pm 23.7	0.10 to 145.5	61	34.0 \pm 30.4	0.20 to 110.0
Body mass index (kg/meter ²) ^b	749	28.0 \pm 3.8	16.7 to 51.3	82	28.4 \pm 3.8	19.6 to 41.5
Serum triglycerides (mg/dL) ^b	743	151.2 \pm 93.9	24.0 to 978.0	83	146.5 \pm 60.9	49.0 to 340.0
Total serum cholesterol (mg/dL) ^b	753	230.5 \pm 38.7	130.0 to 438.0	83	232.7 \pm 32.6	158.0 to 297.0
Serum high-density lipids (mg/dL) ^{b*}	730	49.4 \pm 13.3	21 to 131	83	45.8 \pm 10.3	21.0 to 85.0
Alcohol intake (gm/day) ^b	737	13.3 \pm 17.5	0.0 to 104.1	80	11.2 \pm 14.6	0.0 to 67.0
Systolic blood pressure (mm Hg) ^b	753	134.9 \pm 17.0	91.0 to 215.0	83	136.4 \pm 18.6	103.0 to 186.0
Diastolic blood pressure (mm Hg) ^b	753	82.0 \pm 9.3	51.0 to 122.0	83	81.5 \pm 11.3	56.0 to 110.0
Diabetes	754	81 (10.7)	—	83	9 (10.8)	—
Hypertension	754	341 (45.2)	—	83	42 (50.6)	—
Family history of hypertension	635	277 (43.6)	—	72	30 (41.7)	—
Person time (years) ^{b*}	754	6.9 \pm 2.3	1.8 to 10.4	83	3.8 \pm 2.7	0.08 to 10.7
Blood lead ($\mu\text{g}/\text{dL}$) ^b	738	6.2 \pm 4.3	0.0 to 35.0	80	7.0 \pm 3.8	1.0 to 20.0
Blood lead* tertiles						
< 5 $\mu\text{g}/\text{dL}$	738	306 (41.5)	—	64	22 (27.5)	—
5–9.9 $\mu\text{g}/\text{dL}$	738	329 (44.6)	—	64	43 (53.8)	—
≥ 10 $\mu\text{g}/\text{dL}$	738	103 (14.0)	—	64	15 (18.8)	—
Patella lead ($\mu\text{g}/\text{g}$) ^{b*}	487	30.6 \pm 19.7	–10.0 to 165.0	63	36.8 \pm 20.8	5.0 to 101.0
Patella lead ($\mu\text{g}/\text{g}$) ^{b*} tertiles						
Tertile 1	487	13.9 \pm 4.9	–10.0 to 20.0	63	15.3 \pm 4.3	5.0 to 19.0
Tertile 2	487	27.1 \pm 4.1	21.0 to 34.0	63	25.7 \pm 3.8	21.0 to 33.0
Tertile 3	487	52.5 \pm 20.7	35.0 to 165.0	63	53.3 \pm 17.3	35.0 to 101.0
Tibia lead ($\mu\text{g}/\text{g}$) ^b	486	21.4 \pm 13.6	–3.0 to 126.0	63	24.2 \pm 15.9	–5.0 to 75.0
Tibia lead ($\mu\text{g}/\text{g}$) ^b tertiles						
Tertile 1	486	10.2 \pm 3.8	–3.0 to 15.0	63	10.1 \pm 5.3	–5.0 to 15.0
Tertile 2	486	19.1 \pm 2.3	16.0 to 23.0	63	19.8 \pm 2.2	16.0 to 23.0
Tertile 3	486	35.5 \pm 14.4	24.0 to 126.0	63	39.5 \pm 14.9	25.0 to 75.0

^aTotal n for the respective variable. ^bMean \pm SD. * $p < 0.05$ for cases versus noncases. ** $p < 0.10$ for cases versus noncases.

Martin et al. 2006; Moller and Kristensen 1992; Nash et al. 2003; Pirkle et al. 1985; Schwartz 1991, 1995). Furthermore, it has also been reported that higher blood lead levels lead to increased mortality from cardiovascular causes (Lustberg and Silbergeld 2002). However, only three previous investigations have assessed the association between blood lead levels and heart disease (Kromhout 1988; Moller and Kristensen 1992; Pocock et al. 1988). Pocock et al. (1988) followed 7,371 men 40–59 years of age in Britain for 6 years, to assess the relationship between blood lead levels at baseline and IHD. Although mean blood lead concentration was significantly higher in cases (0.786 $\mu\text{mole/L}$) than in non-cases (0.735 $\mu\text{mole/L}$), there was no evidence that blood lead was associated with IHD after controlling for potential confounders. Moller and Kristensen (1992) studied the risk of fatal and nonfatal coronary heart disease and cardiovascular disease in 1,050 participants after 14 years of follow-up. Their results were similar to those of Pocock et al. in that blood lead was associated with increased risk for coronary heart disease (relative hazard = 2.14; $p = 0.003$) and cardiovascular disease (relative hazard = 1.58; $p = 0.05$) in an unadjusted analysis; but the association disappeared when confounders were adjusted for. Another smaller study ($n = 141$) by Kromhout (1988) in the Netherlands found no association between blood lead and coronary heart disease in univariate and multivariate analysis. However, only 26 participants had coronary heart disease in their 8 years of follow-up data. A recent case report described a patient with angina (severe spontaneous chest pain with S-T elevation) who had a normal coronary angiogram and blood lead level of 33 $\mu\text{g/dL}$ (Oneglia et al. 1998). The patient was chelated with EDTA, and described to be normal during follow-up. The authors hypothesized that lead exposure was possibly involved in endothelial dysfunction and coronary spasm in this case.

It is likely that previous studies (Kromhout 1988; Pocock et al. 1988), although suggestive of a relationship between lead exposure and

heart disease, did not find an association in multivariate analysis because of differences in study population. Another likely reason is that blood lead was used as a biomarker for exposure. Lead accumulates in the skeleton, with a half-life of years to decades (Manton 1985; Rabinowitz et al. 1976). Bone is a repository for 90–95% of lead in adults (Barry and Mossman 1970; Saltzman et al. 1990; Schroeder and Tipton 1968). Previous studies have shown that bone lead levels remain elevated despite declines in blood lead. Therefore, bone lead may be the biomarker of choice for measurement of long-term lead exposure. Bone lead levels have been found to be better predictors than blood lead when assessing outcomes such as hypertension and cognitive declines in a number of recent studies (Cheng et al. 2001; Hu et al. 1996a; Schwartz et al. 2000; Weisskopf et al. 2004). There is evidence that lead is released from bone stores, especially during increased bone turnover (Rabinowitz 1991; Silbergeld 1991). This may contribute to increased blood lead in persons with increased bone lead or increased bone turnover.

Blood and bone lead were associated with increased risk for IHD in our study. Furthermore, the effect estimates of blood and bone lead were not attenuated when assessed simultaneously, suggesting that both contribute independently to IHD. It is unclear why tibia lead was not significantly associated with IHD, although the direction of association was consistent with our overall findings. The stronger association of patella lead with IHD is noteworthy in that the patella is composed of trabecular bone and is known to have higher turnover rates and contribute more to blood lead than the cortical bone represented by tibia lead (Hu et al. 1996b). Because bone lead may contribute to blood lead, particularly in our aging cohort, which has had greater historic environmental exposures and higher rates of bone resorption, the association of blood lead with IHD is plausible. It is also likely that persons in the general population with high blood lead levels have historically had higher levels of lead. In

summary, blood lead level reflects acute exposure from circulating lead, whereas bone lead reflects chronic exposure as well as the major internal source of circulating blood lead. Both factors likely play a role in predicting risk for IHD. We suggest that future studies look at both blood and bone lead when assessing the risk for IHD from lead exposure.

The pathogenesis of the association between lead exposure and IHD can be explained by two mechanisms: One is mediation through increase in blood pressure, which has been previously associated with an increase in risk for ischemic and coronary heart disease (Khot et al. 2003; MacMahon et al. 1990; Tibblin et al. 1975; Wojtczak-Jaroszowa and Kubow 1989); and the other is by atherogenic process. Atherosclerosis can result from lead exposure by inhibition of cytochrome P-450, leading to accumulation of lipids in vessel walls. Lead exposure can also lead to inhibition of superoxide dismutase, an oxygen radical-scavenging enzyme, leading to an increase in serum lipid peroxide (Moller and Kristensen 1992; Wojtczak-Jaroszowa and Kubow 1989). Serum peroxide is a risk factor for vascular disease and thrombus formation.

Although lead levels have declined in the United States and other industrialized nations, low-level lead exposures still persist, and exposure from higher lead levels in the past is likely. Because the pathogenesis of IHD is chronic and takes years to develop, the public health implications of cumulative lifetime lead exposure in the general population are likely being currently realized and will continue in the near future.

Our study was limited by the unavailability of exact date of onset for the IHD event. Therefore, 31 December of the year of IHD diagnosis was used in person-time calculations. However, it is unlikely that this would lead to a differential bias by IHD status. Because our study population included only men and had very few minority participants, our results may not be generalized to races other than white or to women. Our study also had a limited number of IHD events. Therefore, residual

Table 2. Cox proportional hazards models for the association between biomarkers of lead level and IHD, Normative Aging Study, 1991–2001 [HR (95% CI)].

Covariate	Unadjusted	Model A ($n = 787$)	Model B ($n = 787$)	Model C ($n = 532$)	Model D ($n = 531$)
Age (years)					
< 60	Reference	Reference	Reference	Reference	Reference
60–69	2.18 (1.10–4.32)	2.43 (1.19–4.97)	2.45 (1.20–5.03)	1.67 (0.77–3.64)	1.71 (0.78–3.76)
≥ 70	2.44 (1.16–5.10)	2.52 (1.15–5.49)	2.57 (1.18–5.61)	2.01 (0.83–4.84)	2.22 (0.91–5.42)
Black race	2.38 (0.87–6.49)	1.84 (0.58–5.90)	1.71 (0.53–5.53)	1.99 (0.61–6.45)	2.14 (0.66–6.94)
Serum high-density lipids (mg/dL)	0.97 (0.96–0.99)	0.97 (0.95–0.99)	0.97 (0.95–0.99)	0.98 (0.96–1.00)	0.98 (0.96–1.00)
Blood lead level ≥ 5 $\mu\text{g/dL}$	1.64 (1.00–2.68)	1.73 (1.05–2.87)*	—	—	—
Blood lead level ($\mu\text{g/dL}$) ^a	1.40 (0.99–1.98)	—	1.45 (1.01–2.06)*	—	—
Patella lead level ($\mu\text{g/g}$) ^a	3.27 (1.41–7.58)	—	—	2.64 (1.09–6.37)*	—
Tibia lead level ($\mu\text{g/g}$) ^a	2.76 (0.94–8.12)	—	—	—	1.84 (0.57–5.90)**

HR, hazard ratio; CI, confidence interval. The hazard ratios and their statistical significance for blood and bone lead were similar when other potential confounders such as smoking, body mass index, alcohol consumption, blood pressure, family history of hypertension, and total serum cholesterol were included in the models.

^aLogarithm of lead level. * $p = 0.05$; ** $p = 0.31$.

confounding unaccounted for in our analysis is a possibility. This includes factors such as measures of socioeconomic status that are related to lead levels. A lower socioeconomic status may lead to inadequate health maintenance, thereby increasing the risk for IHD.

Conclusion

In summary, we found that men with increased blood and bone lead levels were at an increased risk for future IHD. Low-level lead exposures in the recent past and higher past exposures may contribute to the increased risk for IHD. Although, the pathogenesis of IHD is multifactorial, lead exposure may be one of the risk factors for development of IHD.

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Concentrations of Urinary Phthalate Metabolites Are Associated with Increased Waist Circumference and Insulin Resistance in Adult U.S. Males

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BACKGROUND: Phthalates impair rodent testicular function and have been associated with anti-androgenic effects in humans, including decreased testosterone levels. Low testosterone in adult human males has been associated with increased prevalence of obesity, insulin resistance, and diabetes.

OBJECTIVES: Our objective in this study was to investigate phthalate exposure and its associations with abdominal obesity and insulin resistance.

METHODS: Subjects were adult U.S. male participants in the National Health and Nutrition Examination Survey (NHANES) 1999–2002. We modeled six phthalate metabolites with prevalent exposure and known or suspected antiandrogenic activity as predictors of waist circumference and log-transformed homeostatic model assessment (HOMA; a measure of insulin resistance) using multiple linear regression, adjusted for age, race/ethnicity, fat and total calorie consumption, physical activity level, serum cotinine, and urine creatinine (model 1); and adjusted for model 1 covariates plus measures of renal and hepatic function (model 2). Metabolites were mono-butyl phthalates (MBP), mono-ethyl phthalate (MEP), mono-(2-ethyl)-hexyl phthalate (MEHP), mono-benzyl phthalate (MBzP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP).

RESULTS: In model 1, four metabolites were associated with increased waist circumference (MBzP, MEHHP, MEOHP, and MEP; *p*-values ≤ 0.013) and three with increased HOMA (MBP, MBzP, and MEP; *p*-values ≤ 0.011). When we also adjusted for renal and hepatic function, parameter estimates declined but all significant results remained so except HOMA-MBP.

CONCLUSIONS: In this national cross-section of U.S. men, concentrations of several prevalent phthalate metabolites showed statistically significant correlations with abdominal obesity and insulin resistance. If confirmed by longitudinal studies, our findings would suggest that exposure to these phthalates may contribute to the population burden of obesity, insulin resistance, and related clinical disorders.

KEY WORDS: androgens, homeostatic model assessment, insulin resistance, obesity, phthalates. *Environ Health Perspect* 115:876–882 (2007). doi:10.1289/ehp.9882 available via <http://dx.doi.org/> [Online 14 March 2007]

Obesity, insulin resistance, and type 2 diabetes are interrelated metabolic disorders whose prevalence has increased substantially in the past two decades. Corresponding increases in premature morbidity and mortality are expected (Adams et al. 2006; Fujimoto 2000; Haffner et al. 1998; Poirier et al. 2006; Zimmet et al. 2001). Insulin resistance occurs when increasing amounts of insulin are required to correctly regulate transport of plasma glucose into peripheral tissues. Although the precise mechanism is unclear, insulin resistance is commonly accompanied by central (visceral) obesity, which, by elevating levels of free fatty acids in serum, may provoke insulin resistance and disrupt lipid metabolism. Initially, the beta cells of the pancreas can fully compensate for mild insulin resistance by increasing insulin production. As the disease progresses, beta cells decompensate, resulting in elevated serum glucose levels and the subsequent development of type 2 diabetes.

Testosterone affects body fat distribution and insulin sensitivity in men. Experimental studies in males have shown that testosterone

administration reduces lipid uptake by intra-abdominal fat (Mårin et al. 1996) and also reduces visceral fat and improves insulin sensitivity (Mårin 1995; Mårin et al. 1992, 1993). A 2005 meta-analysis found that testosterone administration reduces total fat mass (Isidori et al. 2005). Men undergoing androgen deprivation therapy for prostate cancer have increased serum glucose, total fat, and prevalence of metabolic syndrome (Braga-Basaria et al. 2006; Sharif et al. 2005). Epidemiologic studies often support these findings (Ding et al. 2006; Selvin et al. 2007), but sometimes they do not (Oh et al. 2002).

Humans are commonly exposed to man-made chemicals that have the potential to reduce androgen (e.g., testosterone) production or function. One such class of chemicals is phthalates, which are used in a variety of products, including cosmetics, shampoos, soaps, lubricants, pesticides, and paints; it is also used as a softener of polyvinyl chloride. More than 75% of the U.S. population has measurable levels of several phthalate metabolites in the urine (Silva et al. 2004). Unlike polychlorinated biphenyls (PCBs) and dioxins,

phthalates are quickly metabolized and excreted (Hauser and Calafat 2005). The half-life of di(2-ethylhexyl)phthalate (DEHP), one of the most widely used and studied phthalates, is < 24 hr (Koch et al. 2004).

Phthalates are known antiandrogens in experimental animal models, with consistent results dating back several decades. Testicular steroid hormone synthesis and reproductive system development in males have been adversely affected by exposure, especially neonatal exposure, to certain phthalates, including DEHP, di-butyl phthalate (DBP), benzyl-butyl phthalate, and di-isononyl phthalate (Bell 1982; Fisher 2004; Parks et al. 2000).

Associations between certain phthalate metabolites and antiandrogenic effects have also been found in humans at much lower exposure levels than those used in rodent experiments. Suspected metabolites include mono-benzyl phthalate (MBzP), mono-ethyl phthalate (MEP), mono-isononyl phthalate (MiNP), mono-methyl phthalate, and mono-butyl phthalate (MBP). Urinary phthalate metabolites in pregnant women have been found to correlate with subtle genital changes in their infant males (Swan et al. 2005), and breast-milk phthalate metabolites have been correlated with shifts in reproductive hormones in infant males (Main et al. 2006).

Although fetuses and infants are thought to be more susceptible to environmental insult than adults, Duty et al. (2003) and Hauser et al. (2006) found diminished sperm quality associated with urinary phthalate metabolites in adult males as well. If their findings reflect true antiandrogenic effects of phthalates or their metabolites at current exposure levels, then one may reasonably predict that these exposures could increase the prevalence of metabolic disorders that are worsened by diminished androgen production or function.

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We thank D. Fernandez, P. Winters, and P. Auinger for their study-design advice; J. DeWester for alerting R.W.S. to links between testosterone, obesity, and diabetes in the mid-1990s; and our anonymous reviewers for their thoughtful comments.

The authors declare they have no competing financial interests.

Received 3 November 2006; accepted 1 March 2007.

In this study we examined the association between phthalate exposure and two key metabolic abnormalities associated with hypogonadism: abdominal obesity and insulin resistance. Although these conditions are closely related, they represent key precursors—alone or in combination—to the development of type 2 diabetes and cardiovascular disease (Janssen et al. 2004; Reaven 1988). Our hypothesis was that increased phthalate exposure would be associated with increased abdominal obesity and insulin resistance.

Methods

Study population. We used data from the 1999–2002 National Health and Nutrition Examination Survey (NHANES) for this analysis. NHANES, conducted by the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention (CDC), is a multistage, stratified, clustered design that selects a representative sample of the civilian, noninstitutionalized U.S. population. Certain subgroups, such as older adults, Mexican Americans, non-Hispanic blacks, and low-income persons, were sampled at a higher rate than other demographic groups, thus necessitating the use of sample weights in analysis. Data from NHANES subjects are acquired through household interviews and standardized examinations at mobile examination centers throughout the United States. Detailed methods have been published elsewhere (NCHS 2006b).

We limited our analysis to men > 18 years of age with complete data for the measures described below. Men on insulin, oral hypoglycemic agents, or sex hormone agonists/antagonists were excluded because these medications may affect the biological mechanisms of interest. Men were also excluded from the insulin resistance analyses if they reported to NCHS that they failed to fast for 8–24 hr before collection of fasting blood samples.

In NHANES 1999–2002, a random one-third subsample was selected for urinary phthalate metabolite measurements, and a separate random, but overlapping, one-third subsample was selected for fasting glucose and insulin (used to compute insulin resistance) measurements. Of the 5,094 adult men available in NHANES, 1,451 men had both phthalate and obesity measurements for crude analyses after exclusions. Of these men, 45% ($n = 651$) also had fasting measurements. Two of the phthalate metabolites, mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), were only available for the years 2001–2002, reducing sample size for these metabolites by about half (waist circumference, $n = 781$; insulin resistance, $n = 344$). Missing data reduced sample sizes for fully

adjusted models (vs. crude) by 11% for waist circumference and 4.5% for insulin resistance.

Abdominal obesity. Waist circumference was chosen as the best available measure of abdominal obesity. As a predictor of insulin resistance, waist circumference has been found to be an equivalent and, in some cases, a better measure than body mass index (Farin et al. 2006; Janssen et al. 2004). Waist circumference was measured at the high point of the iliac crest at minimal respiration to the nearest 1 mm.

Insulin resistance. We estimated insulin resistance using HOMA (homeostatic model assessment). HOMA is epidemiologically practical, widely used, and correlates acceptably ($R = 0.73$ – 0.88) with the hyperinsulinemic-euglycemic clamp test, which is generally considered to be the gold standard (Matthews et al. 1985; Wallace et al. 2004). HOMA was calculated from fasting plasma glucose and insulin measures following the method of Matthews et al. (1985):

$$\text{HOMA} = [\text{fasting insulin } (\mu\text{U/mL}) \times \text{fasting glucose } (\text{mmol/L})] / 22.5. \quad [1]$$

Plasma glucose was determined by an enzymatic reaction (Cobas Miras assay); plasma insulin was determined using a radioimmunoassay with the double-antibody batch method.

Phthalate exposure. Phthalate data were collected in NHANES as urinary metabolites—rather than unmetabolized phthalates in serum—to eliminate contamination during collection and analysis (Latini 2005). Laboratory methods have been previously described (Silva et al. 2004). Seven metabolites were measured throughout the 4-year period, with five additional metabolites measured in 2001–2002 subjects only. For measurements below the limit of detection (LOD), the NCHS assigned a default value of LOD divided by the square root of 2, a method of handling nondetectable values that produces reasonably nonbiased means and SDs (Hornung and Reed 1990).

Of the seven phthalates with data for all 4 years, four (MBP, MEP, MiNP, MBP) are suspected human antiandrogens based on existing human studies (Duty et al. 2003; Hauser et al. 2006; Main et al. 2006; Swan et al. 2005). Metabolites of DEHP, the most widely studied antiandrogenic phthalate, were also of interest: mono-(2-ethylhexyl) phthalate (MEHP) because it was available for all 4 years, and MEHHP and MEOHP (2001–2002 only) because secondary DEHP metabolites are suspected to be more biologically active (Koch et al. 2005; Strohecker et al. 2005). MiNP was eliminated from further consideration because its concentration was < LOD in > 75% of subjects. In NHANES 1999–2000, MBP represented both mono-*n*-butyl phthalate and mono-isobutyl

phthalate, whereas in NHANES 2001–2002, these metabolites were measured separately (CDC 2005). In the present study, we summed the two mono-butyl phthalate metabolites in 2001–2002 data to permit analyses of mono-butyl phthalates over the 4-year period.

Potential confounders. Covariates included in our analyses were age, race/ethnicity, family history of diabetes, dietary fat and caloric intake, physical activity, income, renal function, hepatic function, and exposure to tobacco smoke. Race/ethnicity was self-identified (white, black, Mexican American, other Hispanic, and other/multiethnic). Family history of diabetes was dichotomous (yes, no). Total dietary fat (continuous) and caloric intake (continuous) were computed from a 24-hr recall dietary questionnaire. Two physical activity measures were included: moderate to vigorous leisure activity (continuous; metabolic equivalents/month) and video-based (computer, video, TV) inactivity (categorical, hours/day: 0, < 1, 1, 2, 3, 4, ≥ 5). Socioeconomic status was represented as a “poverty income ratio,” a measure of income relative to family size and compared to the federal poverty threshold (categorical, percentage of poverty threshold: < 100, 100–199, 200–299, 300–399, 400–499, > 500). The renal function measure was glomerular filtration rate (GFR; continuous), estimated using the four-variable equation from the Modification of Diet in Renal Disease study (Levey et al. 1999), incorporating serum creatinine adjustments per NCHS instructions (NCHS 2006c). Liver function was represented by alanine aminotransferase (ALT; continuous) and gamma glutamyl transferase (GGT; continuous). Exposure to tobacco smoke was determined using serum cotinine (continuous).

Statistical analysis. For descriptive analyses, we computed median and mean phthalate metabolite levels, adjusting for urine concentration by dividing metabolite measurements by urine creatinine. These analyses were conducted for the entire sample, and also stratified by race/ethnicity and age.

Linear regression analyses were performed with HOMA (log-transformed) and waist circumference as outcome variables. Because several phthalate metabolites are strongly correlated, each urinary phthalate metabolite was examined separately. Phthalate metabolite concentrations were log-transformed to normalize the data. Categorical analyses by exposure quintiles were also performed.

We conducted crude analyses and two adjusted analyses for each phthalate. Adjusted model 1 includes covariates discussed below. Adjusted model 2 includes these covariates, plus GFR, ALT, and GGT. Model 1 was our primary model because phthalates are known to affect the liver (Bhattacharya et al. 2005;

Lapinskas et al. 2005; Rusyn et al. 2006); therefore, adjusting for liver function (model 2) could remove true effects. Model 2 is also needed, however, because obesity can affect liver function (Lawlor et al. 2005) and thus alter phthalate metabolism. GFR had minimal effect on results but was left in model 2 for completeness.

Age and race/ethnicity are known confounders and were included in fully adjusted models. Urine creatinine was included to correct for urine concentration, as recommended by Barr et al. (2005). Because the relationship between age and prevalence of metabolic syndrome (an outcome related to our outcomes) appears curvilinear (Park et al. 2003), age² was also included. Age, age², urine creatinine, and race/ethnicity were forced into all models regardless of their influence on metabolite regression coefficients. Other covariates were evaluated as possible confounders.

Covariates whose removal caused metabolite parameter estimates to change by ≥ 10% were considered confounders and left in the adjusted models (Greenland 1989). Food intake (total fat and calories), serum cotinine, and activity measures were confounders for two or more metabolites, and were therefore included as covariates. Family history of diabetes and poverty income ratio were not important confounders and had many missing values; thus they were omitted. The sample size for the full regression model was reduced by 11% for waist circumference and 4.5% for insulin resistance due to missing data for one or more covariates.

To assess the contribution of phthalate metabolites to the model fit, the percent variation in the outcome measures explained by each metabolite was calculated by computing the difference in adjusted R² between the full model with and without that metabolite.

To convert regression coefficients to clinically interpretable measures, we first computed the absolute change in waist circumference and log HOMA represented by

an increase in the significant (*p* ≤ 0.05) log phthalate metabolites from the 10th to 90th percentiles, then calculated this increase in waist circumference and HOMA as a percent of their medians:

$$\text{Percent change in waist circumference (at median)} = \frac{\beta \times [\ln(\text{metabolite}_{90\text{th percentile}}) - \ln(\text{metabolite}_{10\text{th percentile}})]}{\text{median waist circumference}} \quad [2]$$

$$\text{Percent change in HOMA (at median)} = \frac{\exp\{\beta \times [\ln(\text{metabolite}_{90\text{th percentile}}) - \ln(\text{metabolite}_{10\text{th percentile}})]\}}{\text{median HOMA}} \quad [3]$$

where β is the regression coefficient for each individual metabolite.

We used SAS, version 9.1 (SAS Institute, Cary, NC) for all statistical analyses. Appropriate weight variables were used to account for oversampling of special demographic groups in NHANES, and sampling cluster variables were used for its complex

sampling design. Phthalate subsample weight variables were used for both waist circumference and HOMA analyses. Because we examined only the adult male subpopulation in the NHANES data, we used subpopulation methods as described by Graubard and Korn (1996).

The use of weights associated with individual NHANES subsamples may be inaccurate when two subsamples that do not completely overlap are used simultaneously in an analysis (NCHS 2006a). Our waist circumference analyses used only one subsample (phthalates) and are unaffected; however, HOMA analyses used both fasting and phthalate subsamples, which only overlap by 50%. Consequently, the use of phthalate weights in our HOMA analyses could affect validity of these findings. Nevertheless, use of fasting subsample weights, phthalate weights, and no weights did not substantially alter our interpretation of the HOMA results, which also demonstrated similar patterns as our

Table 2. Association between waist circumference, HOMA (ln), and selected phthalate metabolites (ln): NHANES 1999–2002.

Outcome	Crude analysis		Adjusted model 1 ^a		Adjusted model 2 ^b	
	β (SE)	p-Value	β (SE)	p-Value	β (SE)	p-Value
Waist circumference						
1999–2002 (n = 1,451 crude, 1,292 adjusted)						
MBP	1.39 (0.51)	0.011	0.98 (0.50)	0.059	0.79 (0.47)	0.106
MBzP	1.18 (0.47)	0.017	1.29 (0.34)	0.001	1.09 (0.36)	0.005
MEHP	0.24 (0.40)	0.550	0.62 (0.44)	0.170	0.53 (0.42)	0.217
MEP	0.95 (0.32)	0.005	0.77 (0.29)	0.013	0.66 (0.31)	0.041
2001–2002 (n = 781 crude, 696 adjusted)						
MEHHP	1.82 (0.58)	0.007	1.71 (0.56)	0.008	1.65 (0.50)	0.005
MEOHP	2.00 (0.63)	0.006	1.81 (0.60)	0.009	1.79 (0.55)	0.005
HOMA (ln)						
1999–2002 (n = 651 crude, 622 adjusted)						
MBP	0.061 (0.024)	0.016	0.064 (0.024)	0.011	0.043 (0.023)	0.081
MBzP	0.059 (0.027)	0.037	0.079 (0.023)	0.002	0.061 (0.022)	0.009
MEHP	0.035 (0.023)	0.143	0.031 (0.025)	0.225	0.016 (0.024)	0.526
MEP	0.067 (0.021)	0.004	0.056 (0.020)	0.008	0.044 (0.021)	0.045
2001–2002 (n = 344 crude, 327 adjusted)						
MEHHP	0.054 (0.029)	0.078	0.055 (0.028)	0.064	0.038 (0.023)	0.126
MEOHP	0.066 (0.031)	0.052	0.060 (0.032)	0.076	0.044 (0.027)	0.125

^aAdjusted for age, age², race/ethnicity, total fat and calorie intake, physical activity level, smoking exposure, and urine creatinine. ^bAdjusted for model 1 covariates plus GFR, ALT, and GGT.

Table 1. Mean median phthalate serum metabolite concentrations (μg/g creatinine): NHANES 1999–2002.^a

	No.	4-Year				2-Year		
		MBP	MBzP	MEHP	MEP	MEHHP	MEOHP	
		Mean ± SE (median)	Mean ± SE (median)	Mean ± SE (median)	Mean ± SE (median)	Mean ± SE (median)	Mean ± SE (median)	
All	1,443	33.8 ± 1.6 (21.2)	29.4 ± 2.7 (14.2)	11 ± 1.3 (3.8)	771 ± 66.7 (188.1)	780	65.8 ± 7.9 (19.6)	38.7 ± 4.5 (13.2)
By age (years)								
19–35	469	36.1 ± 2.9 (23.9)	35.7 ± 6.0 (17.0)	12.8 ± 2.2 (5.1)	699.2 ± 117.2 (212.4)	253	77.5 ± 18.2 (22.1)	45.8 ± 9.9 (15.6)
36–50	386	31.0 ± 2.1 (21.4)	25.0 ± 1.8 (14.9)	13.4 ± 2.7 (3.9)	672.1 ± 130.3 (173.5)	219	68.9 ± 12.7 (20.1)	39.3 ± 8.2 (13.2)
51–65	284	33.4 ± 3.2 (18.3)	25.1 ± 4.3 (11.8)	7.1 ± 1.3 (2.5)	1017.5 ± 160.8 (208.7)	160	52.8 ± 14.4 (16.3)	32.1 ± 7.7 (11.6)
66–80	231	37.9 ± 5.7 (18.4)	25.9 ± 4.3 (11.3)	5.9 ± 1.0 (2.2)	933.7 ± 182.7 (139.1)	118	42.8 ± 17.5 (11.5)	26.0 ± 8.8 (7.5)
≥ 81	73	24.2 ± 2.9 (19.0)	49.6 ± 31.9 (12.1)	3.7 ± 0.7 (2.4)	503.7 ± 180.9 (94.8)	30	20.3 ± 4.9 (13.2)	13.5 ± 3.3 (8.3)
By ethnicity								
Mexican American	335	33.1 ± 4.1 (23.2)	24.3 ± 3.3 (12.7)	8.6 ± 1.1 (4.0)	717.0 ± 82.2 (259.6)	161	34.0 ± 7.7 (15.6)	20.6 ± 4.2 (10.8)
Other Hispanic	75	39.7 ± 6.9 (24.2)	29.3 ± 4.1 (13.8)	14.9 ± 4.5 (6.9)	1347.3 ± 338.0 (292.1)	35	48.8 ± 17.3 (25.3)	30.0 ± 10.5 (15.9)
White	717	32.0 ± 1.9 (19.0)	28.5 ± 3.3 (14.0)	10.9 ± 1.8 (3.3)	703.1 ± 72.9 (158.8)	415	70.2 ± 11.8 (19.5)	41.9 ± 6.3 (13.1)
Black	271	45.6 ± 4.4 (31.7)	44.0 ± 9.4 (19.9)	13.4 ± 3.0 (5.3)	1086.5 ± 177.1 (362.6)	150	83.0 ± 24.7 (32.1)	42.5 ± 9.0 (18.7)
Other/multiethnic	45	28.7 ± 3.9 (28.1)	18.8 ± 2.8 (11.9)	5.0 ± .8 (3.8)	295.8 ± 65.6 (84.6)	19	14.7 ± 3.4 (12.3)	9.7 ± 2.1 (9.2)

^a2001–2002 only for MEHHP and MEOHP.

waist circumference analyses. This suggests that the use of two partially overlapping subsamples did not significantly impact our findings.

Results

Table 1 shows the median and mean phthalate levels in the U.S. population, overall and stratified by age and race/ethnicity. Exposure levels vary widely by phthalate metabolite, with MEHP having the lowest concentration among the six we analyzed, and MEP the highest. Concentrations varied somewhat by age, with greater median levels usually found in younger age groups. For all phthalate metabolites except MEHP, > 95% of subjects were at \geq LOD; for MEHP, 80% of subjects were \geq LOD.

Concentrations also varied by race/ethnicity. Blacks had higher levels of exposure than whites and Mexican Americans for all phthalate metabolites. Mexican Americans had somewhat higher levels than whites for MBP, MEP, and MEHP.

Table 2 shows the regression results for crude analysis (adjusting only for urinary creatinine) and fully adjusted models. In the adjusted model 1, increasing concentrations of MBzP, MEHHP, and MEOHP were statistically significantly associated with greater waist circumference, whereas concentrations of MBP, MBzP, and MEP were significantly associated with increased log HOMA. Adjusted model 2, which adjusted for renal and hepatic function, showed declines in parameter estimates, but all significant results remained so except HOMA-MBP.

Adjusted model 1 HOMA results gave similar parameter estimates whether calculated with phthalate or fasting subsample weights (< 5% difference for analyses with significant p -values). For further verification, an unweighted HOMA analysis was also conducted (data not shown) with somewhat larger changes in parameter estimates. However, MBP, MEP, and MBzP were still significantly associated and MEHP was again not significant. MEHHP and MEOHP gained significance in the unweighted analysis.

Categorical dose-response analyses demonstrated that the assumption of linearity was not strongly violated, although the curves appeared to level off, or perhaps decline, at higher metabolite concentrations (Figure 1).

The contribution of phthalate metabolites to model fit is displayed in Table 3. Adjusted R^2 of the full model ranged from 15 to 20% for both outcome measures. The addition of significant phthalate metabolites explained 0.4–2.1% of outcome variability. Compared with the overall explanatory power of the full model, individual metabolites contributed between 2.5 and 10.1% of the model fit.

To convert regression coefficients to clinically interpretable measures, we computed the

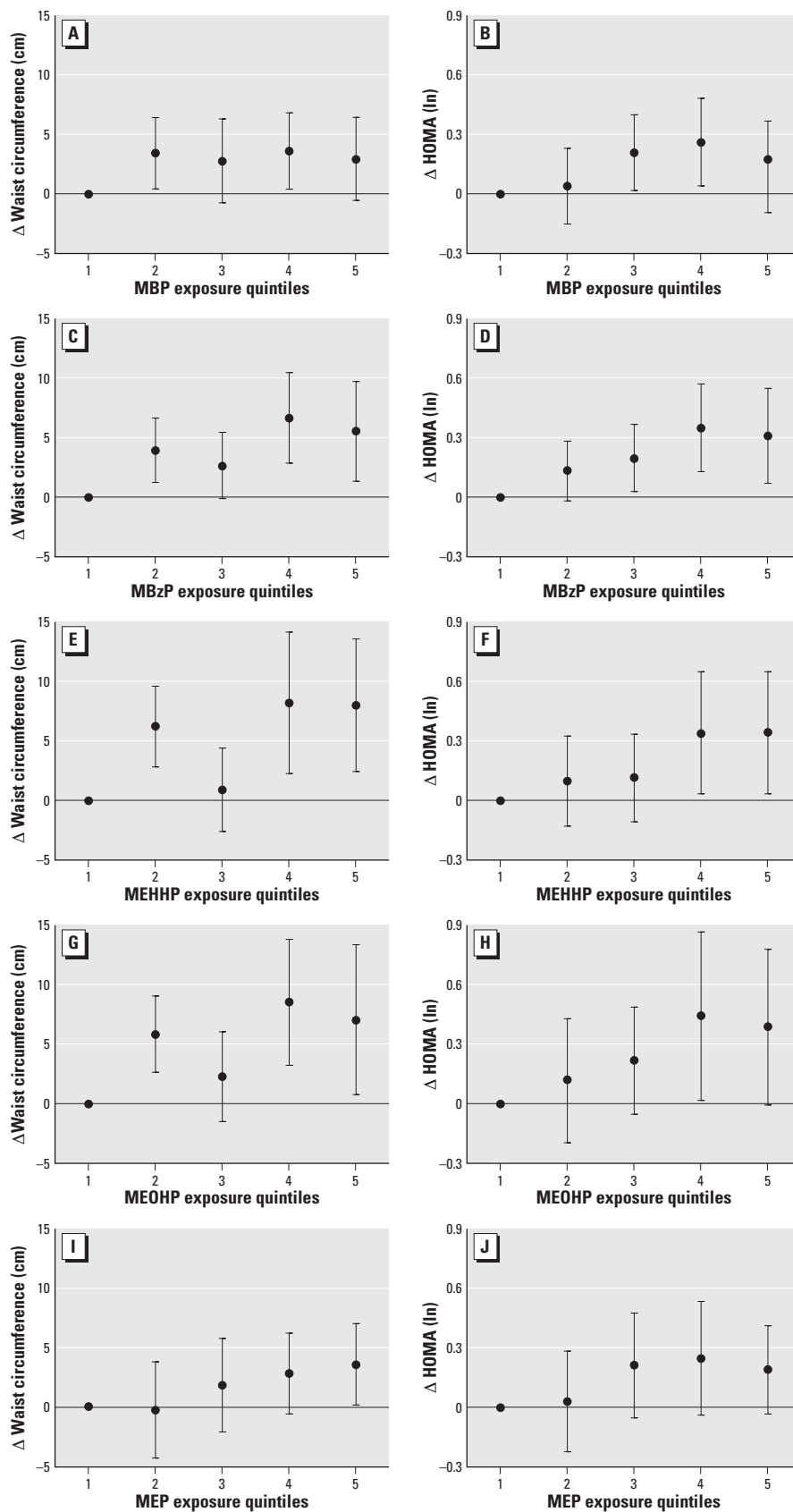


Figure 1. Fully-adjusted associations (adjusted model 1) between changes in outcomes and exposure quintiles (quintile 1 is reference) for metabolites with significant/near-significant ($p \leq 0.10$) continuous regression coefficients. Error bars indicate 95% confidence intervals.

change in waist circumference and HOMA represented by an increase in significant phthalate metabolites from the 10th to 90th percentiles in adjusted model 1. Waist circumference increased 3.9–7.8 cm (4.0–8.0% of the 97.0-cm median) for four significant metabolites: MEP (3.9 cm), MBzP (5.8 cm), MEHHP (7.3 cm), and MEOHP (7.8 cm). HOMA (at the 2.50 median) increased 1.3–1.4 (52–57% of median) in association with three metabolites: MBP (1.3), MEP (1.3), and MBzP (1.4).

Discussion

Obesity, insulin resistance, and diabetes have increased substantially in prevalence over the past three decades. Many plausible causes have been described, such as perinatal exposures, reduced physical activity, food marketing strategies, poor city planning, and thrifty genes (Ebbeling et al. 2002). However, if bioavailable testosterone levels in American men have declined considerably in recent years, as recently reported by Travison et al. (2007), then hypoandrogenism could be another contributor to the epidemic of obesity and related disorders. Hormonally active agents such as phthalates could be one source of this decline in testosterone level or, perhaps independently, to a decline in androgen function.

In the present study, we found that the log-transformed concentrations of several phthalate metabolites were positively and significantly correlated with abdominal obesity (MBzP, MEHHP, MEOHP, MEP) and insulin resistance (MBP, MBzP, MEP) in adult U.S. males. Categorical analysis of these metabolites by exposure quintiles yielded dose-response curves consistent with this interpretation. Although wide confidence intervals preclude strong assertions, the HOMA analyses suggest the inverted-U shaped, non-monotonic dose-response sometimes seen with hormonally active agents, including phthalates (Andrade et al. 2006; Lehmann et al. 2004; Takano et al. 2006; Welshons et al. 2003).

Adjustment for renal function had minimal effect. Adjustment for liver function moderated most associations but only eliminated

statistical significance for HOMA-MBP. Whether this adjustment for liver function was entirely appropriate is unclear, however. Adjustment could be appropriate because obesity can affect liver function, and thus may alter phthalate metabolism. Conversely, adjusting for liver function could falsely attenuate real effects if phthalate exposure was partially responsible for elevation of liver enzymes.

Among the DEHP metabolites available, MEHP showed substantially weaker associations compared with the oxidative metabolites MEHHP and MEOHP. This was not unexpected because a recent study showed MEHHP and MEOHP more active in animal models than MEHP (Stroheker et al. 2005). Further, MEHP has a shorter serum half-life than these other DEHP metabolites, thus reducing the correlation of MEHP measurements with DEHP exposure (Koch et al. 2005).

To our knowledge, ours is the first human study to examine associations between phthalate metabolites and either abdominal obesity or insulin resistance. In one animal study, however, female rats exposed to DEHP were found to have increased serum glucose and decreased insulin, as well as thyroid and adrenocortical dysfunction (Gayathri et al. 2004). However, the decreased insulin in that study points to impaired beta cell function in the pancreas, as found in type 1 diabetes or later in the course of type 2 diabetes.

Like our study, others have found associations with outcomes that might follow from antiandrogenic effects of MBP (Duty et al. 2003; Hauser et al. 2006; Main et al. 2006; Swan et al. 2005) and MEP (Jönsson et al. 2005; Main et al. 2006; Swan et al. 2005), and found no association with MEHP (Duty et al. 2003; Hauser et al. 2006; Jönsson et al. 2005; Main et al. 2006; Swan et al. 2005). Results for MBzP, MEHHP, and MEOHP were less consistent. Assuming these associations represent true effects, differences between our results and others could be due to our larger sample sizes and increased power. Phthalates might also affect adult males differently than fetuses, or they may interact

directly with energy balance or glucose metabolism pathways in addition to antiandrogenic effects.

In our study, individual phthalate exposures only explained $\leq 2.1\%$ of the variability of our outcomes (abdominal obesity and insulin resistance). The full model, however, explained only 15–20%, despite including the well-accepted covariates of age, race/ethnicity, fat and calorie intake, activity levels, and smoking. In part, this demonstrates the difficulty of predicting the presence of this complex, multifactorial clinical syndrome. In addition, humans are exposed to multiple phthalates simultaneously and in combination with other potential environmental toxicants that may add together to produce adverse effects. For example, PCBs, dioxins, and organochlorine pesticides have also been associated with diabetes prevalence in adult humans (Lee et al. 2006; Rylander et al. 2005). Combinations of phthalates and other potential antiandrogens have been shown in animal models to act in a dose-additive manner (Gray et al. 2006; Hotchkiss et al. 2004); in humans, Hauser et al. (2005) found greater than additive effects between MBP and PCB-153 on semen quality. Although individual phthalate exposures in humans are generally asserted to be below the no observed effect level (NOEL), combinations of man-made estrogenic chemicals, individually below NOEL concentrations, have been shown to exert considerable effect in a yeast reporter gene assay using the human estrogen receptor- α (Rajapakse et al. 2002).

Estrogenic exposures might also add to antiandrogenic exposures; some authors have found the estradiol/testosterone (total) ratio correlated more strongly with fasting glucose and insulin than testosterone (free or total) or estradiol alone (Phillips et al. 2003). Among xenoestrogens, bisphenol A (currently unmeasured in NHANES) would be important to investigate because it has recently been shown to affect insulin production in mouse pancreatic beta cells in a manner similar to that of estrogen (Alonso-Magdalena et al. 2006).

Our findings should be considered in light of several important limitations. First, as a cross-sectional study, this design cannot examine changes over time. Second, HOMA is a static measure of insulin resistance, unlike the hyperinsulinemic–euglycemic clamp, which limits its ability to detect abnormalities in insulin secretion or peripheral glucose disposal. This may have reduced our ability to observe associations. Third, NHANES 1999–2002 contains no measures of sex hormones, gonadotropins, or sex hormone-binding globulin in men, which limits our ability to examine the mechanism of action proposed in this study. Fourth, our HOMA analyses are less generalizable to the population because specially calculated sample weights

Table 3. Outcome variation explained by and contribution to fit of phthalate metabolites significantly ($p \leq 0.05$) associated with one or both outcome measures (adjusted model 1).

	Adj R^2 of full model with metabolite	Adj R^2 of full model without metabolite	Outcome variation explained by metabolite (%) ^a	Model contribution of metabolite (%) ^b
Waist circumference				
MBzP	0.1587	0.1517	0.7	4.4
MEHHP	0.2042	0.1837	2.1	10.0
MEOHP	0.2043	0.1837	2.1	10.1
MEP	0.1556	0.1517	0.4	2.5
HOMA (ln)				
MBP	0.1650	0.1575	0.8	4.5
MBzP	0.1748	0.1575	1.7	9.9
MEP	0.1727	0.1575	1.5	8.8

Adj, adjusted.

^a $(R^2_{\text{with metabolite}} - R^2_{\text{without metabolite}}) \times 100\%$. ^b $[(R^2_{\text{with metabolite}} - R^2_{\text{without metabolite}}) / R^2_{\text{with metabolite}}] \times 100\%$.

for the combined use of phthalate and fasting subsamples are not available.

Fifth, our study was restricted to adult males. Children and adolescents were excluded from our study because fasting glucose and insulin were not available in NHANES 1999–2002 for subjects < 12 years of age, and because in adolescents, hormone levels vary greatly with stage of puberty. Women were excluded due to the high degree of premenopausal fluctuations in sex hormones and the change in risk profile at menopause. Also, women appear to respond differently than men to low testosterone levels; in women, low testosterone has been associated with reduced prevalence of obesity, insulin resistance, and diabetes (Ding et al. 2006; Kalish et al. 2003; Oh et al. 2002). An analysis of the adult women in this data set indeed gave dissimilar results from men (results not shown) and deserves further study.

Sixth, the one-time spot urine samples used in this study are limited measures of long-term exposure to phthalates. If phthalates are exerting antiandrogenic effects, one might expect this effect to manifest over months to years. No study has yet examined the correlation between a spot urine phthalate measurement and year-long exposure patterns. Hauser et al. (2004b), however, found that a single spot urine predicted the highest tercile of phthalate exposure over 3 months with sensitivities from 0.56 to 0.67 and specificities from 0.83 to 0.87 among the phthalates in our study. Hauser et al. also noted that urine creatinine may not be the best way to correct for variation in urine dilution, and that specific gravity (unavailable in NHANES) may be a better approach. These flaws in exposure classification also reduced our ability to observe associations.

Seventh, people with obesity or insulin resistance may be exposed to more phthalates than people who do not have these conditions. A few Food and Drug Administration–licensed medications incorporate phthalates to modify drug delivery (Schettler 2006), and this exposure can sometimes be quite large (Hauser et al. 2004a). Because people with disease are also more likely to take medications, some of the associations we have observed, particularly with MBP and MEP (metabolites of DBP and diethyl phthalate), may be falsely strengthened through reverse causality. Adjustment for this possible confounder would be difficult, however. Phthalates are considered inert ingredients, and we are not aware of a comprehensive database of inert ingredients for prescription and over-the-counter medications, herbals, and vitamins.

The present study has several important strengths, based primarily on strengths of NHANES data. Our sample was large, nationally representative, and multiethnic.

Biomarkers were used for both exposures and outcomes, and a number of relevant covariates could be controlled.

Although we based our study on the premise that phthalates are acting as antiandrogens, the relationships between phthalate metabolites, abdominal obesity, and insulin resistance may be complicated by other mechanisms. For example, phthalates can act as thyroid hormone receptor antagonists (Sugiyama et al. 2005). Some phthalate metabolites are also known to interact with peroxisome proliferator–activated receptors (PPARs), which are not only important regulators of lipid and glucose homeostasis (Evans et al. 2004) but also mediate some effects of phthalates on testicular and hepatic function (Corton and Lapinskas 2005; Lapinskas et al. 2005). Activation of PPARs appear to have generally beneficial effects on lipid and glucose homeostasis, though medications that activate PPAR γ improve insulin sensitivity while simultaneously increasing nonvisceral fat mass (Semple et al. 2006).

Several research paths would help determine the importance of our findings. Animal studies conducted in human exposure ranges, as well as human cross-sectional studies, could explore the capacity of combinations of hormonally active agents to create metabolic disturbances such as those we have examined here. Studies similar to ours could be extended to women, children, and adolescents, or to related conditions such as metabolic syndrome. Measurement of sex hormones, unavailable for males in NHANES 1999–2002, might suggest possible mechanisms. Examination of lipid effects could help tease out PPAR contributions. Ultimately, longitudinal studies will be required to provide more definitive answers.

In conclusion, in this large national cross-sectional sample, several phthalate metabolites showed statistically significant positive correlations with abdominal obesity and insulin resistance in adult U.S. males. If confirmed by longitudinal studies, these associations would suggest that phthalates, a widely used family of chemicals, may contribute to the prevalence of obesity, insulin resistance, and related clinical disorders. Because phthalates are rapidly metabolized, unlike PCBs and other persistent organic contaminants, such confirmation could prompt effective actions to reduce phthalate exposure in the population.

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Positive Associations of Serum Concentration of Polychlorinated Biphenyls or Organochlorine Pesticides with Self-reported Arthritis, Especially Rheumatoid Type, in Women

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BACKGROUND: Persistent organic pollutants (POPs) can influence the immune system, possibly increasing the risk of rheumatoid arthritis (RA). In addition, as metabolic change due to obesity has been proposed as one mechanism of osteoarthritis (OA), POPs stored in adipose tissue may be also associated with OA.

OBJECTIVE: Our goal in this study was to examine associations of background exposure to POPs with arthritis among the general population.

DESIGN: We investigated cross-sectional associations of serum POPs concentrations with the prevalence of self-reported arthritis in 1,721 adults ≥ 20 years of age in the National Health and Nutrition Examination Survey 1999–2002.

RESULTS: Among several POPs, dioxin-like polychlorinated biphenyls (PCBs) or nondioxin-like PCBs were positively associated with arthritis in women. After adjusting for possible confounders, odds ratios (ORs) were 1.0, 2.1, 3.5, and 2.9 across quartiles of dioxin-like PCBs (p for trend = 0.02). Corresponding figures for nondioxin-like PCBs were 1.0, 1.6, 2.6, and 2.5 (p for trend = 0.02). Organochlorine (OC) pesticides were also weakly associated with arthritis in women. For subtypes of arthritis, respectively, RA was more strongly associated with PCBs than was OA. The adjusted ORs for RA were 1.0, 7.6, 6.1, and 8.5 for dioxin-like PCBs (p for trend = 0.05), 1.0, 2.2, 4.4, and 5.4 for nondioxin-like PCBs (p for trend < 0.01), and 1.0, 2.8, 2.7, and 3.5 for OC pesticides (p for trend = 0.15). POPs in men did not show any clear relation with arthritis.

CONCLUSIONS: The possibility that background exposure to PCBs may be involved in pathogenesis of arthritis, especially RA, in women should be investigated in prospective studies.

KEY WORDS: arthritis, persistent organic pollutants, pesticides, polychlorinated biphenyls, rheumatoid arthritis. *Environ Health Perspect* 115:883–888 (2007). doi:10.1289/ehp.9887 available via <http://dx.doi.org/> [Online 20 February 2007]

Persistent organic pollutants (POPs) are organic chemical compounds that are highly toxic, persist in the environment, bioaccumulate in fatty tissues of living organisms, travel long distances, and naturally flow toward colder climates (Abelsohn et al. 2002). Humans are generally exposed to POPs through their food supply (Abelsohn et al. 2002).

Whether the exposure to endocrine disruptors such as POPs at current background environmental levels is harmful to human health has become a matter of intense debate, politically and scientifically (Kaiser 2000). However, we recently reported striking associations of serum concentrations of several POPs with diabetes in a random sample of the general population with background exposure to POPs (Lee et al. 2006b). In a recent editorial (Lee et al. 2006a), we discussed that selection of a reference group with a known very low exposure is critical in the estimation of POPs-associated risks. This is because risks of several POPs-associated conditions appear to increase substantially even within a narrow range of low POPs concentrations, not detectable without substantial blood volume. Thus, epidemiologic studies on POPs in the general population

could identify strong associations that might have been missed in previous epidemiologic studies in people exposed to high concentrations of selected POPs that used the general population as the reference group, as if its substantial range of exposure had uniform risk.

In the present study we hypothesized that background environmental exposure to POPs is also involved in pathogenesis of arthritis. Among the various subtypes of arthritis, rheumatoid arthritis (RA) and osteoarthritis (OA) are the two most common in the general population (Abyad and Boyer 1992). RA is an autoimmune disease in which an as yet unknown trigger results in a chronic inflammatory process affecting the synovial membrane of the joints (Gabriel 2001), while OA is often thought to result from natural aging processes on the joint surfaces (Sharma et al. 2006). Although much is known about the pathophysiology of these conditions at a cellular level, there is considerably less information about the etiology of RA and OA in general population studies.

Endocrine disruptors such as POPs markedly influence the immune system (Ahmed 2000), which could increase the risk of autoimmune diseases such as RA. In

addition, beyond the negative effects of increased weight bearing caused by obesity, metabolic change due to adipose tissue has recently been proposed as one underlying mechanism of osteoarthritis (Dumond et al. 2003). Because POPs stored in adipose tissue can be related to differentiation, metabolism, and function in adipose tissue (Mullerova and Kopecky 2006), POPs could be involved in the relation between obesity and OA. Furthermore, the relations of POPs with arthritis may be different depending on sex because endocrine disruptors such as POPs may exert their effects through sex hormone-related receptors (Crews et al. 2000).

The population-based National Health and Nutrition Examination Survey (NHANES) 1999–2002 measured background concentrations of a variety of POPs. This survey also obtained self-report of clinical diagnosis of history of arthritis and subtypes of arthritis. Although the validity of report of all types of arthritis combined is high, validity of the subtype of arthritis based on questionnaire has been reported to be low (Star et al. 1996). Given this fact, the predominance of RA and OA among all arthritis types, and our hypothesis that jointly involved RA and OA, we primarily focused our investigation on the association between serum concentrations of POPs and prevalence of all arthritis and further analyzed by the subtype of arthritis.

Materials and Methods

The 1999–2002 NHANES conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention (CDC) was designed to be nationally representative of the noninstitutionalized U.S. civilian population on the basis of a complex, multistage probability sample. Details of the NHANES protocol and all testing

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This study was supported in part by the Brain Korea 21 project in 2007.

The authors declare they have no competing financial interests.

Received 7 November 2006; accepted 20 February 2007.

procedures are available elsewhere (NCHS 2006a, 2006b). Serum concentrations of biologically important POPs or their metabolites were measured in subsamples of the NHANES 1999–2002 (NCHS 2005). The study protocol was reviewed and approved by the CDC institutional review board; additionally, informed written consent was obtained from all subjects before they took part in the study.

The NHANES standardized home interview was followed by a detailed physical examination in a mobile evaluation clinic or the participant's home (NCHS 2006a, 2006b). Information about existing medical conditions was collected using questionnaires. Venous blood samples were collected and shipped weekly at –20°C. POPs were measured by high-resolution gas chromatography/high-resolution mass spectrometry using isotope

dilution for quantification. All of these analytes were measured in approximately 5 mL serum using a modification of the method of Turner et al. (1997). Ability to detect low POPs concentrations was greater in those participants who provided a larger aliquot. The POPs were reported on a lipid-adjusted basis using concentrations of serum total cholesterol and triglycerides.

Although 49 POPs were measured in both NHANES 1999–2000 and 2001–2002, to avoid bias in estimation among those below the limit of detection (LOD) we selected the 19 POPs for which at least 60% of study subjects had concentrations > LOD; 3 polychlorinated dibenzo-*p*-dioxins (PCDDs), 3 polychlorinated dibenzofurans (PCDFs), 4 dioxin-like polychlorinated biphenyls (PCBs), 5 nondioxin-like PCBs, and 4 organochlorine (OC) pesticides. A total of 1,721 study participants were ≥ 20 years of age with information available on serum concentrations of the 19 selected POPs.

For each POP, subjects with serum concentrations < LOD were regarded as the reference group, and subjects with detectable values were categorized into quartiles by cutoff points of 25th, 50th, and 75th values. To yield a cumulative measure (which would provide a cumulation of risk across POPs with similar chemical and physical properties) of 3 PCDDs, we summed the rank of 3 POPs that belong to the PCDDs. The summary values were categorized into quartiles by cutoff points of 25th, 50th, and 75th values. We categorized and cumulated POP subclasses similarly for the 3 PCDFs, the 4 dioxin-like PCBs, the 5 nondioxin-like PCBs, and the 4 OC pesticides. For example, a subject had rank 0 for all POPs with serum concentrations < LOD. For other POPs with a detectable level, the participant was ranked accordingly. Thus, depending on the sum of ranks of the several POPs belonging to the specific POP subclass under consideration, the subject could be in the lowest quartile or in a higher quartile; however, if all POPs in the subclass were nondetectable, the subject would be placed in the lowest quartile. We chose the current approach because there is no scientific rationale for summing within the POP subclasses to create broader exposure categories.

Participants were considered to have prevalent arthritis if they answered “yes” to the following question: “Has a doctor or other health professional ever told you that you had arthritis?” They were further asked about the type of arthritis. We used logistic regression models to calculate multivariate-adjusted odd ratios (ORs) and 95% confidence intervals (CIs). All analyses were performed separately in men and women. Adjusting variables were age (years), race/ethnicity, poverty income ratio (continuous), body mass index (BMI; continuous), and cigarette smoking (never,

Table 1. Age-adjusted Spearman correlation coefficients^a between five categories of lipid-adjusted POPs (3 PCDDs, 3 PCDFs, 4 dioxin-like PCBs, 5 nondioxin-like PCBs, and 4 OC pesticides) with demographic or health behavior factors by sex.

Characteristic	PCDDs	PCDFs	Dioxin-like PCBs	Nondioxin-like PCBs	OC pesticides
Males					
Age	0.53**	0.35**	0.65**	0.69**	0.72**
Race	NS	0.07*	NS	NS	-0.29**
Poverty income ratio	NS	NS	0.10**	0.10**	-0.17**
BMI	0.24**	0.14**	0.15**	NS	0.18**
Current smoker	-0.14**	NS	-0.13**	NS	NS
Females					
Age	0.60**	0.52**	0.76**	0.73**	0.78**
Race	-0.10**	0.08*	0.08*	0.10**	-0.35**
Poverty income ratio	NS	0.07*	0.09**	0.12**	-0.16**
BMI	NS	NS	NS	-0.17**	NS
Current smoker	-0.19**	NS	NS	0.09**	NS

NS, not significant. For race, white = 1, and others = 0. For current smoker, current = 1, and others = 0.
^aBefore calculating correlation coefficients, detectable values of each POP were individually ranked, and the rank order of the individual POPs in each subclass were summed to arrive at the subclass value; all nondetectable values were ranked as 0. **p* < 0.05. ***p* < 0.01.

Table 2. Adjusted^a OR (95% CI) of prevalence of arthritis by quartiles of PCDDs, PCDFs, dioxin-like PCBs, nondioxin-like PCBs, and OC pesticides in males and females.^b

Analyte	< 25th	25th to < 50th	50th to < 75th	≥ 75th	<i>p</i> _{trend}
Males					
PCDDs					
Cases/participants (no.)	17/191	35/193	47/193	65/192	
Adjusted OR (95% CI)	Referent	1.5 (0.8–2.9)	1.6 (0.8–3.1)	1.4 (0.7–2.8)	0.48
PCDFs					
Cases/participants (no.)	30/192	29/192	43/193	62/192	
Adjusted OR (95% CI)	Referent	0.7 (0.4–1.2)	0.9 (0.5–1.6)	1.1 (0.6–1.9)	0.52
Dioxin-like PCBs					
Cases/participants (no.)	16/192	32/192	47/193	69/192	
Adjusted OR (95% CI)	Referent	1.3 (0.6–2.5)	1.1 (0.6–2.3)	1.3 (0.6–2.8)	0.56
Nondioxin-like PCBs					
Cases/participants (no.)	12/192	24/192	72/193	56/192	
Adjusted OR (95% CI)	Referent	1.2 (0.5–2.6)	3.3 (1.6–6.9)	1.5 (0.7–3.4)	0.23
OC pesticides					
Cases/participants (no.)	14/192	28/192	43/193	62/192	
Adjusted OR (95% CI)	Referent	1.1 (0.5–2.3)	1.5 (0.7–3.2)	1.2 (0.5–2.7)	0.72
Females					
PCDDs					
Cases/participants (no.)	28/238	54/238	62/238	106/238	
Adjusted OR (95% CI)	Referent	1.4 (0.8–2.5)	1.0 (0.6–1.9)	1.3 (0.7–2.4)	0.67
PCDFs					
Cases/participants (no.)	31/237	52/239	63/238	104/238	
Adjusted OR (95% CI)	Referent	1.2 (0.7–2.1)	0.9 (0.5–1.6)	1.3 (0.8–2.3)	0.51
Dioxin-like PCBs					
Cases/participants (no.)	11/237	36/239	91/238	112/238	
Adjusted OR (95% CI)	Referent	2.1 (1.0–4.5)	3.5 (1.7–7.4)	2.9 (1.3–6.5)	0.02
Nondioxin-like PCBs					
Cases/participants (no.)	16/238	34/238	89/238	111/238	
Adjusted OR (95% CI)	Referent	1.6 (0.8–3.1)	2.6 (1.3–5.1)	2.5 (1.2–5.2)	0.02
OC pesticides					
Cases/participants (no.)	19/238	37/238	73/238	121/238	
Adjusted OR (95% CI)	Referent	1.2 (0.6–2.3)	1.3 (0.7–2.6)	1.8 (0.9–3.9)	0.09

^aAdjusted for age, race, poverty income ratio, BMI, and cigarette smoking. ^bDetectable values of each POP were individually ranked, and the rank orders of the individual POPs in each subclass were summed to arrive at the subclass value; all nondetectable values were ranked as 0.

former, or current). We substituted median values of noncases for missing BMI or poverty income ratio in 83 subjects; exclusion of these individuals did not change any conclusions.

We performed all statistical analyses with SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and SUDAAN 9.0 (Research Triangle Institute, Research Triangle Park, NC, USA). Estimates of main results were calculated accounting for stratification and clustering (Korn and Graubard 1991), adjusting for age, race and ethnicity, and poverty income ratio instead of using sample weights; this adjustment is a good compromise between efficiency and bias (Graubard and Korn 1999; Korn and Graubard 1991). Because results were very similar with SAS 9.1 and SUDAAN 9.0, we present the results based on SAS 9.1.

Results

The sample of 1,721 participants was 44.7% male, 47.7% white, and 16.9% current

smokers. Mean \pm SD for age was 49.2 \pm 19.0 years (range 20–85 years). Table 1 shows the associations of five subclasses of POPs with demographic or health behavior factors. Age was the strongest and most important correlate of serum concentrations of all five subclasses of POPs in both sexes, with correlation coefficients ranging from 0.35 to 0.72. White subjects had lower concentrations of OC pesticides in both sexes and lower PCDDs in females, but higher concentrations of PCDFs and PCBs. Those with higher income had lower concentrations of OC pesticides but higher PCBs. Males with higher BMI tended to have higher concentrations of most POPs; however BMI was not associated with POPs among females, except in the inverse association with nondioxin-like PCBs. Current smokers tended to have lower concentrations of most POPs. After adjusting for age, we found positive pairwise correlations among serum concentrations of the five subclasses of

POPs with correlation coefficients from 0.24 to 0.73 in men and 0.19 to 0.77 in women. Women had higher serum concentrations of all five subclasses of POPs with age-adjusted correlation coefficients with sex of 0.22–0.34.

There were 414 prevalent self-reported arthritis cases (164 men and 250 women): 93 RA, 116 OA, 37 other types of arthritis, and 168 unspecified arthritis cases. Neither PCDDs nor PCDFs were associated with arthritis in either sex. However, women who had higher concentrations of dioxin-like PCBs or nondioxin-like PCBs showed a higher risk of prevalence of arthritis (Table 2). After adjusting for age, race/ethnicity, poverty income ratio, BMI, and smoking, ORs were 1.0, 2.1, 3.5, and 2.9 across quartiles of dioxin-like PCBs (p for trend = 0.02). Corresponding figures for nondioxin-like PCBs were 1.0, 1.6, 2.6, and 2.5 (p for trend = 0.02). OC pesticides were weakly associated with the prevalence of arthritis among women;

Table 3. Adjusted^a OR (95% CI) of prevalence of arthritis by categories of specific POPs belonging to dioxin-like PCBs, nondioxin-like PCBs, and OC pesticides in females.

Analyte	Nondetectable	Detectable ^b				p_{trend}
		< 25th	25th to < 50th	50th to < 75th	\geq 75th	
Dioxin-like PCBs						
2,4,4',5-Tetrachlorobiphenyl (PCB-74)						
Cases/participants (no.)	20/334	26/151	53/158	7/154	81/155	
Adjusted OR (95% CI)	Referent	2.1 (1.1–4.1)	2.9 (1.5–5.6)	3.4 (1.8–6.7)	3.2 (1.5–6.7)	< 0.01
2,3',4,4',5-Pentachlorobiphenyl (PCB-118)						
Cases/participants (no.)	21/306	27/162	47/161	74/161	81/162	
Adjusted OR (95% CI)	Referent	1.6 (0.8–3.1)	2.1 (1.1–3.9)	3.0 (1.6–5.8)	2.2 (1.1–4.5)	0.01
3,3',4,4',5-Pentachlorobiphenyl (PCB-126)						
Cases/participants (no.)	26/174	25/193	32/195	70/196	97/194	
Adjusted OR (95% CI)	Referent	0.9 (0.4–1.6)	0.7 (0.4–1.3)	1.3 (0.7–2.3)	1.4 (0.8–2.7)	0.07
3,3',4,4',5,5'-Hexachlorobiphenyl (PCB-169)						
Cases/participants (no.)	26/221	15/183	47/183	76/183	86/182	
Adjusted OR (95% CI)	Referent	0.7 (0.3–1.4)	1.4 (0.8–2.6)	1.6 (0.9–3.0)	1.6 (0.8–3.1)	0.08
Nondioxin-like PCBs						
2,2',3,4,4',5-Hexachlorobiphenyl (PCB-138)						
Cases/participants (no.)	26/233	17/180	52/179	73/181	82/179	
Adjusted OR (95% CI)	Referent	0.9 (0.4–1.8)	1.6 (0.9–2.9)	1.7 (1.0–3.1)	1.3 (0.7–2.5)	0.21
2,2',4,4',5,5'-Hexachlorobiphenyl (PCB-153)						
Cases/participants (no.)	20/199	12/188	50/188	83/189	85/188	
Adjusted OR (95% CI)	Referent	0.7 (0.3–1.5)	1.7 (0.9–3.3)	2.3 (1.2–4.4)	1.6 (0.8–3.2)	0.06
2,2',3,3',4,4',5-Heptachlorobiphenyl (PCB-170)						
Cases/participants (no.)	24/344	36/152	47/152	70/152	73/152	
Adjusted OR (95% CI)	Referent	2.9 (1.6–5.3)	1.9 (1.0–3.6)	2.9 (1.4–5.6)	2.7 (1.3–5.6)	0.03
2,2',3,4,4',5,5'-Heptachlorobiphenyl (PCB-180)						
Cases/participants (no.)	16/234	21/179	48/179	76/181	89/179	
Adjusted OR (95% CI)	Referent	1.4 (0.7–2.9)	2.1 (1.1–4.2)	2.7 (1.3–5.7)	2.7 (1.2–6.0)	< 0.01
2,2',3,4',5,5',6-Heptachlorobiphenyl (PCB-187)						
Cases/participants (no.)	33/396	35/137	46/141	67/140	69/138	
Adjusted OR (95% CI)	Referent	1.9 (1.1–3.3)	1.9 (1.0–3.4)	2.8 (1.5–5.1)	2.2 (1.1–4.3)	0.01
OC pesticides						
<i>p,p'</i> -Dichlorodiphenyldichloroethane						
Cases/participants (no.)	0/0	31/236	44/240	84/239	91/237	
Adjusted OR (95% CI)		Referent	0.9 (0.5–1.6)	1.1 (0.6–2.0)	1.0 (0.5–1.8)	0.93
Oxychlorane						
Cases/participants (no.)	11/185	17/191	51/192	67/192	104/192	
Adjusted OR (95% CI)	Referent	1.1 (0.5–2.6)	2.6 (1.2–5.4)	2.0 (0.9–4.5)	3.1 (1.3–7.1)	< 0.01
<i>trans</i> -Nonachlor						
Cases/participants (no.)	5/104	20/210	47/215	75/212	103/211	
Adjusted OR (95% CI)	Referent	1.4 (0.5–4.0)	2.0 (0.7–5.6)	2.4 (0.9–6.8)	2.2 (0.8–6.6)	0.07
β -Hexachlorocyclohexane						
Cases/participants (no.)	17/225	24/181	58/182	74/182	77/182	
Adjusted OR (95% CI)	Referent	1.0 (0.5–1.9)	1.7 (0.8–3.4)	1.5 (0.7–3.1)	1.4 (0.6–3.2)	0.37

^aAdjusted for age, race, poverty income ratio, BMI, and cigarette smoking. ^bFor each POP, subjects with serum concentrations < LOD were regarded as the reference group.

adjusted ORs were 1.0, 1.2, 1.3, and 1.8 (*p* for trend = 0.09) (Table 2). We also performed analyses adjusting for sex (males and females in one model); adjusted ORs for dioxin-like PCBs were 1.0, 1.6, 2.1, and 2.0 (*p* for trend = 0.02) and those for nondioxin-like PCBs were 1.0, 1.4, 3.0, and 2.1 (*p* for trend < 0.01). However, we found little association among males (Table 2), and we observed significant *p*-values for sex interaction: 0.03 for dioxin-like PCBs and 0.04 for nondioxin-like PCBs. When the study subjects were stratified by age (< 50 and ≥ 50 years) or obesity (BMI < 30 and ≥ 30), the associations were similar to those in Table 2. Most specific POPs belonging to the subclasses of dioxin-like and nondioxin-like PCBs and OC pesticides were positively associated with arthritis among women (Table 3).

For subtypes of arthritis among women, RA (*n* = 93) was more strongly associated with dioxin-like PCBs, nondioxin-like PCBs, or OC pesticides than was OA (*n* = 116) (Table 4). The adjusted ORs (by quartile) for RA were 1.0, 7.6, 6.1, and 8.5 for dioxin-like PCBs (*p* for trend = 0.05), 1.0, 2.2, 4.4, and 5.4 for nondioxin-like PCBs (*p* for trend < 0.01), and 1.0, 2.8, 2.7, and 3.5 for OC pesticides (*p* for trend = 0.15). Adjusted ORs for unspecified arthritis subtype (*n* = 168) were weaker than those of RA but stronger

than those of OA (Table 4), as we expected because these cases were likely a mixture of mostly RA and OA.

In all analyses, we also considered possible confounding by self-reported weight loss in the 1 year or in the 10 years before examination, because weight loss has been reported to increase serum concentrations of POPs (Chevrier et al. 2000). However, the adjustment for weight loss did not materially change the results (data not shown). Additionally, we investigated the associations after excluding subjects with diabetes or cardiovascular diseases, but the results were not different (data not shown).

Discussion

In the present study, background exposure to some kinds of POPs was positively associated with arthritis among women. Especially, among the two most common subtypes of arthritis, RA showed much stronger associations with POPs than did OA, and those associations were of intermediate strength in those with unspecified arthritis type. The validity of self-reported RA is low (Star et al. 1996). However, because subjects did not know their serum levels of POPs and because their exposure to POPs was mainly due to background exposure, nondifferential misclassification is the most likely consequence of

reduced reliability, leading to attenuated strength of association. In this case, null associations could be falsely negative; however, the clear positive associations among women would not be explained by the low validity of RA. In spite of the cross-sectional design, our findings are biologically plausible; this is the first study in the general population with background exposure to POPs. Whether low-dose environmental exposure to POPs in humans could be harmful is one of the most controversial issues in the field of toxicology (Kaiser 2000; Safe 2000; Welshons et al. 2003). However, few epidemiologic studies have been carried out for POPs in the general population, even in a cross-sectional design. The absence of epidemiologic studies in the general population is understandable given the cost of measuring of a variety of POPs and the substantial amount of serum required for their measurement.

Endocrine disruptors such as POPs markedly influence the immune system (Ahmed 2000), but the possibility that the human immune system may respond to a low concentration of POPs has not been studied specifically. However, one might infer such an immune response even to the exposure to the background exposure to POPs on the basis of associations of POPs with diabetes in the general population, which we have reported in this same data set (Lee et al. 2006b). Among the POPs examined in the present study, PCBs were most strongly associated with RA, and the associations did not differ depending on chemical and physical properties of specific PCBs (dioxin-like or nondioxin-like). Interestingly, the known decreasing concentrations of PCBs appear to be consistent with decreasing secular trends of RA over several recent decades in the United States (Alamanos et al. 2006; Doran et al. 2002).

The different associations between PCBs and RA by sex we report here may be biologically plausible because sex hormones appear to play an important role as modulators of autoimmune disease onset and perpetuation, as in the case of RA (Cutolo et al. 2002). Generally, steroid hormones are implicated in the immune response, with estrogens as enhancers at least of the humoral immunity and androgens, progesterone, and glucocorticoids as natural immunosuppressors (Cutolo et al. 2002). Thus, different effects of PCBs by sex may be also possible because endocrine disruptors such as POPs may exert some of their toxicologic effects through sex hormone-related receptors (Crews et al. 2000; Ulbrich and Stahlmann 2004).

Until now, reports of the effects of PCBs on immune function have focused primarily on immunosuppression, with changes in both humoral and cellular immunity (Fernlof et al. 1997; Kimbrough and Krouskas 2001; Lu

Table 4. Adjusted^a OR (95% CI) of prevalence of RA, OA, or unspecified arthritis by categories of dioxin-like PCBs, nondioxin-like PCBs, or OC pesticides in females.^b

Outcome/analyte	< 25th	25th to < 50th	50th to < 75th	≥ 75th	<i>p</i> _{trend}
RA					
Dioxin-like PCBs					
Cases/participants (no.)	2/228	16/219	17/164	26/152	
Adjusted OR (95% CI)	Referent	7.6 (1.7–34.4)	6.1 (1.2–29.7)	8.5 (1.6–44.5)	0.05
Nondioxin-like PCBs					
Cases/participants (no.)	4/226	9/213	21/710	27/154	
Adjusted OR (95% CI)	Referent	2.2 (0.6–7.4)	4.4 (1.3–15.2)	5.4 (1.4–20.3)	< 0.01
OC pesticides					
Cases/participants (no.)	4/223	13/214	19/184	25/142	
Adjusted OR (95% CI)	Referent	2.8 (0.8–8.9)	2.7 (0.7–9.8)	3.5 (0.9–14.0)	0.15
OA					
Dioxin-like PCBs					
Cases/participants (no.)	3/229	12/215	28/175	33/159	
Adjusted OR (95% CI)	Referent	1.9 (0.5–7.2)	2.0 (0.5–7.6)	1.6 (0.4–6.4)	0.97
Nondioxin-like PCBs					
Cases/participants (no.)	4/226	12/216	30/179	30/157	
Adjusted OR (95% CI)	Referent	1.6 (0.5–5.3)	1.7 (0.5–5.8)	1.2 (0.3–4.5)	0.90
OC pesticides					
Cases/participants (no.)	4/223	12/213	22/187	38/155	
Adjusted OR (95% CI)	Referent	1.4 (0.4–4.6)	1.3 (0.4–5.2)	2.1 (0.6–8.2)	0.21
Unspecified arthritis					
Dioxin-like PCBs					
Cases/participants (no.)	5/231	8/211	38/185	44/170	
Adjusted OR (95% CI)	Referent	1.2 (0.4–3.9)	3.8 (1.3–11.2)	2.9 (0.9–9.3)	0.06
Nondioxin-like PCBs					
Cases/participants (no.)	7/229	9/213	36/185	43/170	
Adjusted OR (95% CI)	Referent	1.2 (0.4–3.3)	2.8 (1.0–7.4)	2.8 (1.0–8.3)	0.03
OC pesticides					
Cases/participants (no.)	8/228	10/209	24/188	53/172	
Adjusted OR (95% CI)	Referent	0.8 (0.3–2.3)	1.0 (0.4–2.8)	1.6 (0.6–4.6)	0.14

^aAdjusted for age, race, poverty income ratio, BMI, and cigarette smoking. ^bDetectable values of each POP were individually ranked, and the rank orders of the individual POPs in each subclass were summed to arrive at the subclass value; all nondetectable values were ranked as 0.

and Wu 1985; Nakanishi et al. 1985; Svensson et al. 1994; Van Den Heuvel et al. 2002; Weisglas-Kuperus et al. 2000). Studies on specific clinical correlates have suggested increased prevalence of middle ear (Dewailly et al. 2000; Weisglas-Kuperus et al. 2000) and other bacterial infections (Tsuji 2000). Limited information exists on the potential effects of PCBs on autoimmunity. Most epidemiologic studies on autoimmunity have measured cellular or humoral immunologic function, such as numbers in lymphocyte subpopulations, *in vitro* lymphocyte response, plasma cytokine levels, or autoantibodies. However, the results have not been consistent. Although some studies have reported positive associations (Daniel et al. 2001; Langer et al. 2002), other studies failed to do so (Schoenroth et al. 2004; Tsuji 2000; Tsuji et al. 1999; Yu et al. 1998).

In addition to PCBs in the present study, we found that some pesticides were positively associated with RA among women. Previous studies on the association between pesticides and RA have been inconsistent. Several epidemiologic studies have reported increased RA or increased prevalence of antinuclear antibody among farmers (Lundberg et al. 1994; Olsson et al. 2004; Rosenberg et al. 1999), but others did not (De Roos et al. 2005). Interpretation of previous studies may be limited because the exposure assessment was based on questionnaires about exposure to pesticides rather than on direct measurement of pesticide concentrations in blood.

We were able to evaluate the associations of arthritis with a variety of POPs by taking advantage of the NHANES public use data set. In most epidemiologic studies until now, only a limited set of POPs were evaluated. This approach is sensible when the focus is on occupational or accidental exposure to specific POPs. However, in the study of general populations with background exposure to a mixture of POPs, it may be very important to measure a variety of POPs, because the associations of POPs with health outcomes may differ depending on the specific types of POPs in the exposure and the fact that POPs may interact with each other.

Although the strength of association with OA was weaker than with RA, OA still appeared to be associated with some POPs among women. Recently, metabolic factors related to obesity, such as secretion of leptin, have been linked with the onset and progression of OA (Dumond et al. 2003). Thus, POPs may be involved in the pathogenesis of OA because POPs stored in adipose tissue can cause metabolic disturbances, including over-secretion of leptin (Mullerova and Kopecky 2006). Leptin also plays a key role in a host of autoimmune inflammatory conditions such as RA (Otero et al. 2006).

The present study has several limitations. First, the cross-sectional study design in NHANES does not allow inferences to be drawn regarding temporality of events and the causality between POPs and arthritis. Second, misclassification bias is possible because some subjects with a higher POP value but a lower sample volume could be classified in the reference group, or vice versa. Such misclassification would be nondifferential if sample volume is unrelated to the presence of arthritis. Third, as discussed above, the validity of self-reported RA is low (Star et al. 1996) even though it may be also associated with nondifferential misclassification, leading to attenuated strength of association.

In the present study, serum concentrations of both dioxin-like and nondioxin-like PCBs were positively and possibly nonlinearly associated with arthritis among women. The relations were stronger with RA than with OA. These results raise the possibility that background exposures to some POPs may be involved in the pathogenesis of autoimmune diseases such as RA in women. To confirm these relationships, further study in a prospective cohort study would be necessary.

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Dietary Arsenic Exposure in Bangladesh

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BACKGROUND: Millions of people in Bangladesh are at risk of chronic arsenic toxicity from drinking contaminated groundwater, but little is known about diet as an additional source of As exposure.

METHODS: We employed a duplicate diet survey to quantify daily As intake in 47 women residing in Pabna, Bangladesh. All samples were analyzed for total As, and a subset of 35 samples were measured for inorganic arsenic (iAs) using inductively coupled plasma mass spectrometry equipped with a dynamic reaction cell.

RESULTS: Median daily total As intake was 48 µg As/day [interquartile range (IQR), 33–67] from food and 4 µg As/day (IQR, 2–152) from drinking water. On average, iAs comprised 82% of the total As detected in dietary samples. After adjusting for the estimated inorganic fraction, 34% [95% confidence interval (CI), 21–49%] of all participants exceeded the World Health Organization's provisional tolerable daily intake (PTDI) of 2.1 µg As/kg-day. Two of the 33 women who used a well with < 50 µg As/L exceeded this recommendation.

CONCLUSIONS: When drinking water concentrations exceeded the Bangladesh drinking water standard of 50 µg As/L, ingested water was the dominant source of exposure. However, as drinking water As concentrations decrease, the relative contribution of dietary As sources becomes more important to ingested dose. The combined intake from both diet and drinking water can cause some individuals to exceed the PTDI in spite of using a tube well that contains < 50 µg As/L.

KEY WORDS: arsenic, Bangladesh, dose, duplicate diet, food, intake, water. *Environ Health Perspect* 115:889–893 (2007). doi:10.1289/ehp.9462 available via <http://dx.doi.org/> [Online 20 February 2007]

Use of groundwater has reduced the morbidity and mortality from waterborne disease in Bangladesh and helped the country achieve self-sufficiency in cereal production through dry-season irrigation (Ahmad 2001; Gill et al. 2003). However, the shallow groundwater aquifer of this region is highly contaminated with naturally occurring arsenic from dissolved minerals and ores. In a national survey conducted by the British Geological Survey and the Department of Public Health Engineering, Bangladesh (BGS and DPHE 2001), 27% of the shallow tube wells exceeded the Bangladesh drinking water standard of 50 µg/L, exposing an estimated 33 million people to potentially dangerous levels of As in their drinking water. Chronic exposure to As increases the risk for As-induced diseases such as noncancerous skin lesions, bronchitis, hepatomegaly, neuropathy, peripheral vascular diseases (e.g., gangrene), cardiovascular disease, skin cancer, lung cancer, and bladder cancer (Chen and Ahsan 2004; Chowdhury et al. 2000; Mazumder 2003; McLellan 2002; Smith et al. 1998).

Although there is no question that consumption of As-contaminated drinking water is the most important route of exposure in Bangladesh, little research has focused on food as an additional source of exposure in spite of evidence that rice, a dietary staple, can accumulate As when grown in contaminated environments. Studies have shown that irrigation with As-contaminated water can lead to elevated As concentrations in rice-paddy soil, as well as in the rice root, stalk, and grain (Duxbury et al. 2003; Meharg and

Rahman 2003; Norra et al. 2005). Market basket studies that analyze individual food items also found that As concentrations in commonly consumed vegetables are directly correlated with the As concentration in irrigation water (Alam et al. 2003). Furthermore, cooking with As-contaminated water can be an additional source of exposure because rice absorbs twice its weight in water when cooked (Bae et al. 2002).

To more fully understand the relative contribution of food and drinking water to ingested As dose, we conducted a duplicate diet study in Pabna district, located north of Dhaka in central Bangladesh. We targeted female heads of households because they are responsible for all food preparation; they also represent a potentially susceptible sub-population because there is mounting evidence that As is transmitted to the fetus (Jin et al. 2006) and can influence child neurodevelopment (Wasserman et al. 2004; Wright et al. 2006).

Methods

Participant selection. Forty-seven women who had previously taken part in a longitudinal study investigating As exposure and biomarker response, and who identified themselves as the primary food preparer in the family were invited to participate in this study (Kile et al. 2005). All women agreed to participate, and informed consent was obtained. Two sampling periods were scheduled for 3 consecutive days in winter (January–March 2004) and for 3 consecutive days in summer (June–August

2004). Participants received compensation (US\$9) after each sampling period. The institutional review boards at Harvard School of Public Health and Dhaka Community Hospital approved the protocol for this study.

Food samples. Participants were instructed to save duplicate portions from each meal in separate polypropylene resealable bags. Researchers visited each participant after the midday and evening meals to collect samples, which were kept refrigerated until processing. Each evening, individual portions were weighed in order to determine dietary intake rates. The portions from each participant were then homogenized into a 24-hr composite sample ($n = 282$) using a blender. Homogenized samples were aliquotted into polyethylene tubes and frozen at -4°C and shipped on dry ice.

Field blanks, composed of 50 mL Milli-Q 18.2 Ω analytical grade water (Millipore Corporation, Billerica, MA, USA), were collected after every eighth sample. For the field blanks, the water underwent the same homogenization process as the food samples and was preserved with reagent grade nitric acid (Merck, Darmstadt, Germany) to a pH of < 2. Composite samples were subjected to microwave acid digestion with nitric acid and analyzed for total As using dynamic reaction cell inductively coupled plasma-mass spectrometry (ICP-DRC-MS) with oxygen as the cell gas (Model 6100 DRC; PerkinElmer, Norwalk, CT, USA). This analytical method detects As oxide species ($^{75}\text{As}^{16}\text{O}^{+}$) at mass 91 and avoids argon chloride interference (Bollinger and Schleisman 1999). Indium was added as an internal standard upstream of the nebulizer. The average limit of detection (LOD) was 0.07 µg As/L. The average field blank (\pm SD) contained 0.03 ± 0.07 µg As/L, with only six

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We thank our colleagues, technicians, and laboratory and administrative staff at Dhaka Community Hospital and the Pabna Community Clinic in Bangladesh. We also acknowledge the technical expertise of C. Amarasiriwardena, E. Rodrigues, M. Jones, and E. Madonick, and guidance from R. Wilson and J. Harrington.

This work was supported by grants ES 011622, ES 05947, and ES 00002 from the U.S. National Institutes of Health.

The authors declare they have no competing financial interests.

Received 27 June 2006; accepted 20 February 2007.

field blanks exceeding the average LOD. Each sample was analyzed five times, and the reported total As value was corrected for any As detected in the method blank and field blank. Four samples had corrected total As concentrations below the averaged method LOD of 0.07 µg/L and were assigned half the LOD.

Samples were digested in 14 batches, with each batch containing a method blank, certified rice flour [standard reference material (SRM) 1568A; National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA] and certified dogfish liver sample (DOLT-2; National Research Council, Ottawa, Ontario, Canada). The average percent recovery (\pm SD) was 102.2 \pm 7.9% for SRM 1568A and 93.0 \pm 7.1% for DOLT-2. We used SRM 1643e (Trace Elements in Water; NIST) to validate instrument performance. The average percent recovery for SRM 1643e was 101.7 \pm 5.8%. Additionally, 10% of the samples were randomly chosen for replicate analysis and were analyzed on separate days in the laboratory. The average percent difference in As concentrations detected in replicate samples was 4.0%.

Thirty-five samples representing a range of drinking water exposures were analyzed by Brooks Rand (Seattle, WA, USA) for total As and inorganic arsenic (iAs). This served as an interlaboratory validation for total As and also allowed for the estimation of the iAs fraction in composite dietary samples. Three samples partially thawed during shipping; however, it is unlikely that this brief warming influenced As speciation because it lasted < 24 hr. Brooks Rand extracted the 35 samples for total As and iAs following modifications of U.S. Environmental Protection Agency (EPA) methods 1638 (U.S. EPA 1995b) and 1632 (U.S. EPA 1995a), respectively. For total As, samples were closed-vessel oven-bomb digested with concentrated nitric acid and analyzed by ICP-DRC-MS. For total iAs, sample aliquots were extracted with hydrochloric acid and the pH adjusted to 1.5 before analysis. The comparison between laboratories for total As was good, with an interlaboratory percent difference of 14.6 \pm 19.1%.

The quality control at Brooks Rand included method blanks, certified dogfish

muscle tissue (DORM-2; National Research Council, Canada), dogfish liver tissue (DOLT-3; National Research Council, Canada), and certified lobster hepatopancreas (TORT-1; National Research Council, Canada), spiked reference material, and matrix spikes at concentrations > 10 times the native sample concentration. The percent recovery for DORM-2, DOLT-3, and TORT-1 was 93%, 87%, and 103% respectively. The average percent recovery (\pm SD) for total As from two spiked certified material samples, run in triplicate, was 92.6 \pm 7.3% and the average recovery for iAs was 96.8 \pm 11.9% and 91.5 \pm 4.5%. Water (SRM 1640 and SRM 1643e; NIST) was used to validate instrument performance; the percent recoveries for SRM 1640 and SRM 1643e were 112% and 102%, respectively. All quality assurance met the laboratory's acceptance criteria. The method LODs for total As and iAs were 0.013 and 0.003 µg/g, respectively. All samples were above the LODs.

Drinking water. All women reported having their own tube well and using it for all water for drinking and cooking purposes. Participants were provided with two 4-L polyethylene containers and instructed to place an identical quantity of water in the containers immediately after drinking a glass of water in order to determine drinking water intake rates. However, the concentration of As in each participant's tube well was estimated using water samples collected as part of a larger, longitudinal biomarker study in which water samples were collected for 3 consecutive days every 3 months (Kile et al. 2005). Data from 2004, representing 12 samples per participant and overlapping the time frame of this duplicate diet study, were used to establish an average annual drinking water As concentration for each participant. Total As was measured by Environmental Laboratory Services (North Syracuse, NY, USA) using ICP-MS following U.S. EPA method 200.8 (U.S. EPA 1994).

Quality control criteria included analyzing Plasma CAL#1 Multi-Element QC Standard (SCP Science, Baie D'Urfè, Quebec, Canada). The average percent recovery (\pm SD) was 96.7 \pm 3.4%. Additionally, 10% of the samples were randomly chosen

for replicate analysis, with the average percent difference in As concentrations of 0.2%. Twenty-one households had drinking water As concentrations below the 1-µg/L LOD and were assigned half the LOD.

Statistical analysis. We recorded the weight of each duplicate meal portion (grams wet weight) and the volume of drinking water (milliliters) collected in a 24-hr period as the daily dietary intake rate and drinking water intake rate, respectively. Descriptive statistics were calculated, including mean \pm SD. We calculated daily total As intake from food (micrograms per day) for each participant using the total wet weight of food consumed each day multiplied by the total As concentration measured in the corresponding 24-hr composite sample (micrograms per gram wet weight). We multiplied the annual average total As concentration in each participant's tube well by the total volume of water consumed each day to determine daily total As intake from water (micrograms per day) from drinking water. The daily total As intake was the sum of daily total As intake from food and drinking water. Dividing each participant's daily As intake by their body weight determined the daily total As dose (micrograms per kilogram per day). Medians and interquartile range (IQR; 75th percentile – 25th percentile) were reported for all exposure outcomes.

Because drinking water As concentrations were positively skewed, they were subsequently transformed to their common logarithms. We used generalized estimating equations (GEE) employing an exchangeable working correlation structure to evaluate seasonal and daily differences in As concentrations in food composite samples and dietary and drinking water intake rates. We used two regression techniques, GEE and median regression, to examine the relationship between As-contaminated drinking water and dietary As intake, which approximates the effect of preparing and cooking food with contaminated water. The median regression technique, which is robust to outliers, estimated standard errors using the resampling method while taking into account repeated measures (Parzen et al. 1994).

We estimated the iAs fraction using diet samples with both total As and iAs measured by the same laboratory. The average iAs fraction was then multiplied by the average total As dose from food and summed with the daily As dose from drinking water in order to estimate the amount of iAs ingested. We then compared these values with the World Health Organization's (WHO) provisional tolerable daily intake recommendations (WHO 1985). We report the proportion of participants whose average daily iAs dose exceeded the WHO's PTDI of 2.1 µg/kg-day along with exact confidence intervals (CIs). All statistics were computed using SAS for Windows,

Table 1. Physical and demographic characteristics of the 47 female participants.

Characteristic	Percent of population	Mean \pm SD	Range
Age (years)		36.6 \pm 8.6	20–65
Body mass index		22.5 \pm 3.5	15.1–30.3
Years using tube well		31.9 \pm 7.8	11–54
Years using current tube well		8.7 \pm 6.1	1–20
Marital status			
Married	94		
Widowed	6		
Occupation			
Homemaker	96		
Factory worker	2		
Office worker	2		

version 9.1 (SAS Institute Inc., Cary, NC, USA) except the median regression analysis which was conducted in R, version 2.0.1 (R Foundation for Statistical Computing 2006).

Results

The participant's physical and demographic characteristics are presented in Table 1. The median drinking water concentration for the 47 tube wells sampled was 1.6 $\mu\text{g/L}$ (range, < 1–450 $\mu\text{g/L}$). Overall, 60% were below the WHO's 10- $\mu\text{g/L}$ drinking water standard (WHO 1985), and 70% were below the Bangladesh drinking water standard of 50 $\mu\text{g/L}$ (BGS and DPHE 2001). On average, participants consumed 1,636 g food (wet weight) and 2,676 mL water per day. Participants consumed significantly more food in winter (1,700 \pm 338 g wet weight) than in summer (1,571 \pm 324 g wet weight), but no seasonal difference was detected in the concentration of As in the composite food samples. The number of servings collected did not vary significantly over the course of the study. Also, we did not find a significant difference in the amount of food collected within each season. No seasonal or daily difference was observed in the drinking water intake rate.

The frequency of each food type collected in the duplicate diet study is shown in Table 2. Vegetables and rice were the most commonly consumed food items. Rice, the dietary staple, was present in 91% of all collected meals, with 405 g (wet weight) consumed in an average serving. Vegetables were present in 94% of all meals collected, with an average serving size of 72 g wet weight. Freshwater fish was the most commonly consumed protein. Pabna is far enough inland that seafood is not readily available in the local markets, and no participants reported eating either seafood or shrimp during this study period. Furthermore, all participants reported purchasing their food at local markets. These items would most likely be produced domestically, if not locally. However, this data was not collected.

The distribution of total dietary As intake and dose were heavily skewed, driven by the overwhelming contribution from contaminated drinking water for the upper 25th percentile of the population (Figure 1). When drinking water As concentrations decreased, the relative contribution of As from dietary sources increased. Background dietary total As intake for the population, calculated using the dietary exposures for the participants with no detectable As in their drinking water, was 46 $\mu\text{g/day}$ or 0.91 $\mu\text{g/kg-day}$. For all participants, the combined median daily total As intake from both food and drinking water was 68 $\mu\text{g/day}$ (IQR, 191 $\mu\text{g/day}$). The median daily total As intake from food only was 48 $\mu\text{g/day}$ (IQR, 34 $\mu\text{g/day}$) and drinking water only was 4 $\mu\text{g/day}$ (IQR, 150 $\mu\text{g/day}$).

A subset of 35 samples (12% of the total sample collected) analyzed for both total As and iAs were used to estimate the iAs fraction in the 24-hr dietary composite samples. The average inorganic fraction (\pm SD) in dietary samples was 82.1 \pm 13.9%. Linear regression showed that iAs explained 90% of the variability in total As measurements. To estimate the daily iAs dose, all dietary doses were adjusted by the inorganic fraction before being added to the drinking water doses because it is assumed that all As present in drinking water is in the inorganic form. These values were compared to the WHO's iAs PTDI of 2.1 $\mu\text{g/kg-day}$ (WHO 1985). Overall, 34% (95% CI, 21–49%) of all participants had an average daily dose that exceeded this recommended limit. Of the four women who used

tube wells containing 10–50 $\mu\text{g As/L}$, two exceeded the PTDI. For women who used a tube well containing < 10 $\mu\text{g As/L}$, diet was the only substantial source of ingested As.

Using both GEE and median regression models, we found a significant association between the concentration of As in a given household's drinking water and the total As concentration measured in their food (Figure 2). This likely reflects the effect of cooking and preparing food with As-contaminated water. The median regression model provided the best fit to the average dietary total As intake, as indicated by the smaller SE. This model estimated that dietary total As exposure increased by 0.5 $\mu\text{g/day}$ (95% CI, 0.2–0.7 $\mu\text{g/day}$) for every 10% increase in drinking water As concentration.

Table 2. Frequency of food types collected in the duplicate diet study.

	Winter [No. (%)]	Summer [No. (%)]	Total [No. (%)]
Grains			
Rice	393 (35.0)	390 (33.7)	783 (34.3)
Bread	32 (2.9)	43 (3.7)	75 (3.3)
Proteins			
Fish (all freshwater)	139 (12.4)	95 (8.2)	234 (10.3)
Meat (poultry, beef, goat)	27 (2.4)	21 (1.8)	48 (2.1)
Egg	21 (1.9)	22 (1.9)	43 (1.9)
Fruits and vegetables			
Vegetables	436 (38.8)	374 (32.4)	810 (35.5)
Fruit	1 (0.1)	80 (6.9)	81 (3.6)
Pulses/legumes	65 (5.8)	94 (8.1)	159 (7.0)
Others			
Condiments (sugar, salt)	3 (0.3)	11 (1.0)	14 (0.6)
Fried snacks	2 (0.2)	4 (0.4)	6 (0.3)
Butter	0 (0)	1 (0.1)	1 (0.0)
Dessert (sweet noodles)	2 (0.2)	7 (0.6)	9 (0.4)
Dairy			
Milk ^a	3 (0.3)	14 (1.2)	17 (0.8)

A total of 432 meals were collected for each season, with 864 meals collected in total from 47 participants.

^aWas not included in the 24-hr composite or analyzed for As.

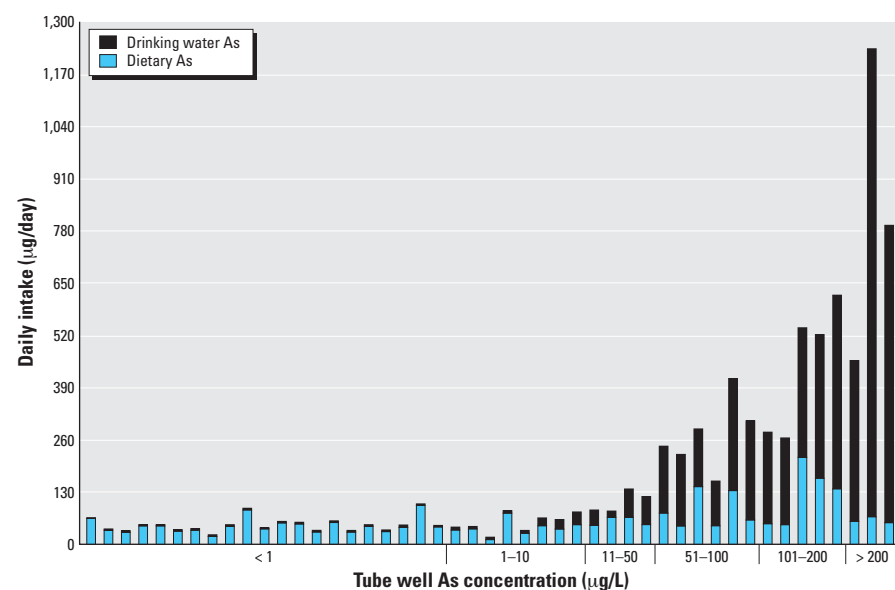


Figure 1. Distribution of average daily As intake ($\mu\text{g/day}$) from both drinking water and dietary sources for all 47 participants sorted by tube well As concentration.

It is interesting to note that only one participant was diagnosed with As-induced skin lesions (melanosis, leukomelanosis, and hyperkeratosis of the palms and soles). This 38-year-old woman reported using the same tube well—one with an average As concentration of 360 µg/L—for the past 12 years. She had the highest observed average daily total As intake (1231.3 µg/day) and subsequent average daily total As dose (25.7 µg/kg-day). However, another participant with no visible As-induced skin lesions had a higher well concentration. This reinforced the notion that interindividual differences in ingestion rates and duration of exposure are an important contributing factor in exposure assessments.

Discussion

In Bangladesh, groundwater provides 95% of the drinking water and approximately 71% of the agricultural irrigation water (Fazal et al. 2001). The shallow aquifer beneath Bangladesh is contaminated with naturally occurring As, and chronic As exposure is widespread throughout the country. Arsenic exposure from drinking contaminated water has received the most attention, primarily because of the high As concentrations detected but also because of the circumstances that generated the current As crisis. In the 1970s, tube wells were installed to switch the population from microbially contaminated surface water to groundwater to decrease the morbidity and mortality from waterborne disease. It was not until 20 years later that the public became aware that these relatively shallow tube wells could be contaminated with As, thus introducing a new health burden on the community.

We observed a median daily total As dose of 1.3 µg/kg-day, whereas the median daily total As dose from drinking water and diet

was 0.08 µg/kg-day and 1.0 µg/kg-day, respectively. These exposure estimates reflect the As contamination in our study area and the relative distribution of As-contaminated water. National groundwater surveys show that As concentrations in approximately 27% of tube wells exceed the Bangladesh drinking water standard of 50 µg/L and 51% exceed the WHO drinking water recommendation of 10 µg/L (BGS and DPHE, 2001). We observed a similar distribution in the present study, with 30% of the tube wells containing > 50 µg/L and 40% containing > 10 µg/L. Our exposure assessment suggested that when tube well concentrations were > 50 µg/L, water was the dominant route of exposure. However, if the observed distribution of contaminated tube wells is representative, then drinking As-contaminated water will be the dominant route of exposure for only one-third of the population. Dietary sources of As, on the other hand, will be the most important route of exposure for the remaining two-thirds of the population. Therefore, it is important to further understand the health risks associated with this route of exposure.

The average daily total As intake calculated in the present study was 174 µg/day, which is considerably lower than the 515 µg/day estimated in an earlier study for an adult Bangladeshi (Watanabe et al. 2004). This discrepancy could be due to regional differences in As contamination or from methodologic differences between the two study designs, because the Watanabe study employed a market basket technique to estimate food-derived exposure and we used a duplicate diet methodology that analyzed As in cooked, ready-to-eat food. Duplicate diet studies are considered to be more accurate at estimating personal exposures because they account for the individual's water source, the type and quantity of food items consumed, and the agricultural conditions under which the food is cultivated (WHO 1985). It is important to note that the estimates derived from duplicate diet studies depend on the dietary habits of the participants and may not be generalizable to other populations. Because we collected dietary data from women only, the results may not be generalizable to men because gender influences the intrafamilial distribution of food in Bangladeshi households, with men eating on average, 40% more cereals, 26% more tubers, 29% more pulses, and 57% more vegetables than women (Hassan and Ahmad 1982). Thus, it is possible that adult males may have higher As exposures than women.

In the present analysis, 34% (95% CI, 21–49%) of the participants ingested iAs concentrations in excess of the WHO's recommended daily allowance of 2.1 µg/kg-day (WHO 1985). If drinking water contained

> 50 µg As/L, water was the dominant route of exposure. However, the combined intake from food and drinking water was sufficiently high that two women who used a well containing 10–50 µg/L exceeded this recommended daily allowance. This provides evidence that the current Bangladesh drinking water standard of 50 µg/L might not be protective of public health when all routes of exposure are considered. The sources of As in the diet are likely to be from rice and vegetables cultivated in As-contaminated environments because these are the two types of food items most commonly consumed. However, we also observed that food preparation modestly contributed to dietary As intake, which has been observed in experimental settings (Bae et al. 2002). However, it is important to recognize that the present study is small, and further studies will be required before determining the source of dietary As and whether the exposure estimates computed for this population are generalizable to other regions in Bangladesh.

It is also important to recognize that the fraction of iAs in food items varies widely (Schoof et al. 1999). We estimated that the average iAs concentration comprised 82% of the total As detected in a subset of the dietary samples. This is similar to values reported by Smith et al. (2006) who reported that iAs made up 87% of the total As measured in rice and 96% of the total As measured in vegetables commonly consumed in Bangladesh. Our estimated inorganic fraction is slightly lower, but we computed the inorganic fraction in homogenized 24-hr dietary samples rather than individual food items. Also, the absorbed dose that influences toxicity depends on the solubility of the iAs during gastrointestinal digestion, which is poorly understood and varies with food type.

Biomarker studies provide evidence that dietary sources contribute to internal dose. Studies that have looked at both urinary and toenail As concentrations found that the relationship between these biomarkers and drinking water As concentrations are nonlinear at low drinking water As concentrations but become linear as drinking water As concentrations increase (Karagas et al. 2000; Kile et al. 2005; Watanabe 2001). It is therefore likely that the added exposure from dietary sources explains the observed nonlinearity in these relationships.

Bangladesh is developing rapidly and has become dependent upon groundwater as a source of drinking and irrigation water. While providing safe drinking water to exposed individuals must remain a public health priority, it is also important that irrigation policies are reviewed, because this analysis clearly demonstrates an elevated background exposure from dietary sources. In accordance with the

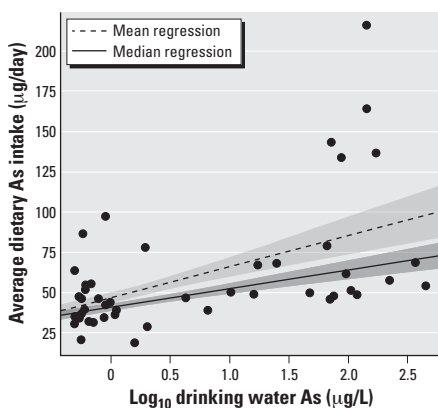


Figure 2. Average dietary As intake (µg/day) plotted against the logarithm of drinking water As (µg/L). The mean and median regressions were obtained from the model $Y = (\alpha + \beta) \times (\log_{10} X + \epsilon)$, where either the mean or median of ϵ is zero. For the median regression line, $\alpha = 40.5$ and $\beta = 11.6$; for the mean regression line, $\alpha = 46.0$ and $\beta = 19.5$. Shaded areas represent 1 SE around each regression line.

recently adopted national policy for As mitigation, which acknowledges that As in groundwater used for irrigation may also have an effect on the food chain, preference should be given to surface water for irrigation where appropriate (Bangladesh Government 2004). Furthermore, it is important to continue to monitor the food chain because continued use of As-contaminated irrigation water is likely to increase the probability and magnitude of dietary As intake.

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PBTK Modeling Demonstrates Contribution of Dermal and Inhalation Exposure Components to End-Exhaled Breath Concentrations of Naphthalene

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BACKGROUND: Dermal and inhalation exposure to jet propulsion fuel 8 (JP-8) have been measured in a few occupational exposure studies. However, a quantitative understanding of the relationship between external exposures and end-exhaled air concentrations has not been described for occupational and environmental exposure scenarios.

OBJECTIVE: Our goal was to construct a physiologically based toxicokinetic (PBTK) model that quantitatively describes the relative contribution of dermal and inhalation exposures to the end-exhaled air concentrations of naphthalene among U.S. Air Force personnel.

METHODS: The PBTK model comprised five compartments representing the stratum corneum, viable epidermis, blood, fat, and other tissues. The parameters were optimized using exclusively human exposure and biological monitoring data.

RESULTS: The optimized values of parameters for naphthalene were *a*) permeability coefficient for the stratum corneum 6.8×10^{-5} cm/hr, *b*) permeability coefficient for the viable epidermis 3.0×10^{-3} cm/hr, *c*) fat:blood partition coefficient 25.6, and *d*) other tissue:blood partition coefficient 5.2. The skin permeability coefficient was comparable to the values estimated from *in vitro* studies. Based on simulations of workers' exposures to JP-8 during aircraft fuel-cell maintenance operations, the median relative contribution of dermal exposure to the end-exhaled breath concentration of naphthalene was 4% (10th percentile 1% and 90th percentile 11%).

CONCLUSIONS: PBTK modeling allowed contributions of the end-exhaled air concentration of naphthalene to be partitioned between dermal and inhalation routes of exposure. Further study of inter- and intraindividual variations in exposure assessment is required to better characterize the toxicokinetic behavior of JP-8 components after occupational and/or environmental exposures.

KEY WORDS: dermal, exposure assessment, inhalation, jet fuel, naphthalene, physiologically based toxicokinetic model. *Environ Health Perspect* 115:894–901 (2007). doi:10.1289/ehp.9778 available via <http://dx.doi.org/> [Online 14 February 2007]

The single largest source of chemical exposure on military bases of the North Atlantic Treaty Organization (NATO) is jet propulsion fuel 8 (JP-8), which is the preferred fuel for both aircraft and military vehicles in NATO countries. JP-8 comprises many aromatic hydrocarbons, including benzene and naphthalene, and aliphatic hydrocarbons such as nonane and decane (McDougal et al. 2000). Exposures to JP-8 can occur during spills, transportation and storage of the fuel, as well as during fueling, general maintenance and operation of aircraft and military vehicles, fueling of military tent heaters, and cleaning and degreasing of parts with the fuel.

Since JP-8 can enter the body via both inhalation and dermal contact, the assessment of occupational exposures to fuel constituents can be difficult. Personal sampling of JP-8 vapors provides information about inhalable levels but not about dermal exposure levels. Similarly, sampling the exposed skin provides information about dermal but not about inhalable levels. Conversely, the collection of end-exhaled breath concentrations provides an integrated estimate of uptake via both inhalation and dermal contact (Egeghy et al. 2003; Pleil et al. 2000) but cannot determine the relative contributions of the two exposure

routes to the internal dose. Through statistical evaluation of levels of naphthalene in air, breath, and skin, measured in the U.S. Air Force personnel during fuel maintenance procedures, both inhalation and dermal exposures to JP-8 were demonstrated to contribute to the internal dose (Chao et al. 2006). However, because of the respiratory protection used in that population, it was difficult to determine the relative contributions of dermal and inhalation exposures to the systemic levels of JP-8 components.

Physiologically based toxicokinetic (PBTK) modeling is an effective tool for quantifying the absorption, distribution, metabolism, and elimination of chemicals. PBTK models have been developed for various components of JP-8, notably naphthalene and decane (Perleberg et al. 2004; Quick and Shuler 1999; Willems et al. 2001). The model developed by Quick and Shuler (1999) focused on the disposition of naphthalene in five compartments representing the lungs, liver, fat, rapidly perfused tissues, and slowly perfused tissues and relied on *in vitro* data to calibrate kinetic constants. Willems et al. (2001) refined the Quick and Shuler (1999) model by using kinetic constants derived from *in vivo* data from laboratory

animal experiments performed by the National Toxicology Program. They observed that a diffusion-limited PBTK model was necessary to characterize the toxicokinetic behavior of naphthalene in rats and mice. Perleberg et al. (2004) developed a PBTK model using decane as a chemical marker of JP-8. Data for calibration and validation of this model were derived from an animal study in which rats were exposed for 4 hr to decane vapor at three different concentrations (1,200, 781, or 273 ppm). Their final model consisted of flow-limited compartments for liver and lung, and diffusion-limited compartments for brain, bone marrow, fat, skin, and spleen. The model predicted the time course of decane in tissue and blood from low-level exposures to decane vapor.

Because the PBTK models mentioned above did not examine the uptake via skin, we developed a PBTK model that included both inhalation and dermal routes of exposure. Naphthalene was chosen as the surrogate for JP-8 exposure because it is abundant in JP-8, is readily absorbed into blood, and is only a minor component in confounding sources of exposure such as cigarette smoke and gasoline exhaust (Rustemeier et al. 2002; Serdar et al. 2003). We expanded on the structure of a data-based compartmental model that was

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We express special thanks to D. Leith and J. Pleil for constructive comments and to L. Ball for allowing the use of her laboratory for the dermal exposure study. We also acknowledge S. Waidyanatha for her assistance with the chemical analysis.

This study was supported by National Institute of Environmental Health Sciences grants P42-ES05948 and T32-ES07018, by the U.S. Air Force through sub-contract 1331/0489-01 with Texas Tech University, and by National Institute for Occupational Safety and Health grant T42/CCT410423-09.

The authors declare they have no competing financial interests.

Received 2 October 2006; accepted 14 February 2007.

used to quantify the absorption, distribution, and elimination of jet fuel components (Kim et al. 2006b). Data from a study of controlled dermal exposure in humans were used to optimize the parameters in the PBTK model (Kim et al. 2006a). The optimal PBTK model, combined with exposure and biomarker data from field studies (Chao et al. 2005; Egeghy et al. 2003), was used to quantify the relative contributions of dermal and inhalation exposures to end-exhaled breath concentrations of naphthalene among U.S. Air Force personnel.

Materials and Methods

Laboratory study of dermal exposure to JP-8. We conducted a laboratory study to quantify the dermal absorption and penetration of JP-8 components across human skin *in vivo* (Kim et al. 2006a). Approval for this study was obtained from the Office of Human Research Ethics (School of Public Health, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina). Written informed consents were received from all study volunteers. The study consisted of 10 volunteers (5 females and 5 males) recruited for this study. Exposures were conducted in an exposure chamber. One forearm was placed palm up inside the exposure chamber, and two aluminum application wells were pressed against the skin and sealed for the duration of the experiment (0.5 hr). At the end of the 0.5-hr exposure period, the exposed sites were tape-stripped 10 times with adhesive tape strips. Tape strips were used to quantify the mass of naphthalene in successive layers of the stratum corneum. Both tape-strip and blood samples were analyzed by gas chromatography–mass spectrometry (GC-MS). The time course of naphthalene in blood for all study volunteers showed considerable interindividual variability. For example, the time course for a 23-year-old Caucasian male with a body mass index (BMI) of 25 kg/m² was very different from that of a 24-year-old Caucasian female with a BMI of 22 kg/m². For the male volunteer, the maximum concentrations in blood (C_{max}) occurred shortly after the end of exposure ($t_{max} \approx 30$ min), with a value of 0.8 ng/mL. The C_{max} for the female volunteer occurred at $t_{max} \approx 60$ min, with a value of 0.3 ng/mL. In either case, the concentrations in blood at $t > 0$ min did not return to baseline levels.

Field study of dermal and inhalation exposures to JP-8. Exposure data were obtained from the assessment of dermal and inhalation exposures to JP-8 in the personnel at six U.S. Air Force bases in the continental United States (Chao et al. 2005; Egeghy et al. 2003). The duration of exposure was approximately 4 hr. The concentration of naphthalene in the personal breathing-zone air (referred to as “air concentration” in this article) was measured

using passive monitors (Egeghy et al. 2003). End-exhaled breath samples were collected pre- and postexposure (Egeghy et al. 2003). The end-exhaled breath measurements are indicative of alveolar air (Egeghy et al. 2000). Both air and breath samples were analyzed by GC with photoionization detection. Tape strips were used to quantify dermal exposure to naphthalene at specific body regions; results were extrapolated to the total surface area of skin to estimate whole-body dermal exposure to naphthalene. We collected dermal samples postexposure using adhesive tape strips with the dimension of 2.5 cm × 4.0 cm (surface area 10 cm²) from exposed body regions including the forehead, neck, shoulders, arms, hands, legs, knees, feet, and buttocks (Chao et al. 2005). Tape-strip samples were extracted with acetone and analyzed by GC-MS.

The median air concentrations of naphthalene in air samples were 1.9 µg/m³ (range, < 1.0–16.9 µg/m³), 29.8 µg/m³ (range, < 1.0–932 µg/m³), and 867 µg/m³ (range, 12.8–3,910 µg/m³) for the low-, medium-, and high-exposure groups, respectively (Egeghy et al. 2003). The median preexposure breath levels of naphthalene were < 0.5 µg/m³ (range, < 0.5–36.3 µg/m³), < 0.5 µg/m³ (range, < 0.5–16.1 µg/m³), and < 0.5 µg/m³ (range, < 0.5–6.1 µg/m³) for the low-, medium-, and high-exposure groups, respectively. The median postexposure breath levels were 0.73 µg/m³ (range, < 0.5–6.9 µg/m³), 0.93 µg/m³ (range, < 0.5–13.0 µg/m³), and 1.83 µg/m³ (range, < 0.5–15.8 µg/m³) for the low-, medium-, and high-exposure groups, respectively. The corresponding median concentrations of dermal samples were 344 ng/m² (range, 159–54,200 ng/m²), 483 ng/m² (range, 150–13,200 ng/m²), and 4188 ng/m² (range, 100–4,880,000 ng/m²) in the low-, medium-, and high-exposure groups, respectively (Chao et al. 2005).

Description of the PBTK model. A dermatotoxicokinetic (DTK) model, which was previously developed for describing the disposition of aromatic and aliphatic components of JP-8 after controlled dermal exposure (Kim et al. 2006b), formed the basis of the PBTK model (Figure 1). The DTK model consisted of five compartments representing the surface, stratum corneum, viable epidermis, blood, and storage tissues. The parameters for the DTK model were estimated by fitting the model to the data. The major difference between the DTK and the PBTK model structures is that the storage compartment was split into fat and all other tissues. The rationale for defining the storage compartment in this fashion was based on the high fat:blood partition coefficient ($P_{f:b}$) of naphthalene (160), which is more than 5 times the partition coefficient of the other tissues (Fiserova-Bergerova 1983). Further additions

to the PBTK model included pulmonary uptake and clearance. The skin compartments were composed of the skin directly under the exposed area. All tissues were perfusion limited and well mixed. Absorbed naphthalene was distributed to other tissue compartments at a rate equal to the rate of blood flow to that tissue. Naphthalene was stored in the fat and other tissue compartments based on the physiologic parameters of that compartment (i.e., tissue:blood partition coefficient, tissue volume, and blood perfusion rate).

Most physiologically based compartmental models separate the arterial blood from the central venous blood, whereas data-based compartmental models treat the blood as one compartment. Also in data-based compartmental models, the peripheral compartments represent organs or tissues that, being poorly perfused with blood, are in slower equilibrium distribution with blood. Blood samples were collected from the antecubital vein in the study by Kim et al. (2006a). The antecubital vein drains blood from the hand and the superficial layers of the forearm. The concentration of

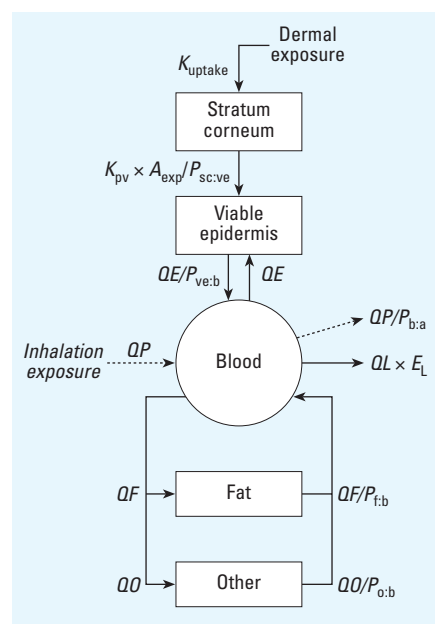


Figure 1. Schematic of the physiologically based toxicokinetic (PBTK) models for the study of naphthalene toxicokinetics. Pulmonary uptake of naphthalene in the personal breathing-zone and pulmonary clearance from the blood compartment are added to a previously published dermatotoxicokinetic model (Kim et al. 2006b). Abbreviations in the PBTK model: K_{uptake} , input rate constant for dermal exposure; K_{pv} , permeability coefficient for the viable epidermis; A_{exp} , exposed surface area; $P_{sc:ve}$, stratum corneum:viable epidermis partition coefficient; QE , blood flow rate to skin; $P_{ve:b}$, viable epidermis:blood partition coefficient; QP , pulmonary ventilation rate; $P_{b:a}$, blood:air partition coefficient; QF , blood flow rate to fat; $P_{f:b}$, fat:blood partition coefficient; QO , blood flow rate to other tissue; $P_{o:b}$, other tissue:blood partition coefficient; E_L , extraction ratio.

solute in the antecubital vein is different from the concentrations of solute in the arterial and central venous blood (Levitt 2004). However, the blood in the antecubital vein is in rapid equilibrium with arterial and central venous blood relative to the fat and other tissue compartments. Therefore, we treated the arterial and central vein as a single compartment, and approximated the concentration of naphthalene in the central (i.e., blood) compartment using measurements made from the antecubital vein.

Two routes of exposure were modeled: dermal and inhalation. Pulmonary uptake is equal to the pulmonary ventilation rate (QP) times the concentration of naphthalene in the personal breathing-zone (C_{PBZ}):

$$\text{Pulmonary uptake} = QP \times C_{PBZ}. \quad [1]$$

In Equation 1, rapid equilibration of naphthalene occurs across the alveolar lining, and neither storage nor metabolism in the lungs appreciably affects the uptake of naphthalene into the systemic circulation. Because arterial, lung, and venous blood are treated as a combined blood compartment, the rate of absorption is equal to pulmonary uptake. Dermal absorption and penetration is modeled as a one-directional diffusive process according to Fick's first law of diffusion. As such, the diffusion of naphthalene across the stratum corneum (SC) and the viable epidermis (VE) are quantified using permeability coefficients, the area of exposure, and the thickness of the membrane (McCarley and Bunge 2001; McDougal and Boeniger 2002). The rate of efflux from the SC to the VE is dependent on the solubility of naphthalene in the SC relative to the VE. Therefore, the rate of efflux of naphthalene from the SC to the VE is equal to $K_{pv} \times A_{exp} \times CD/P_{sc:ve}$, where K_{pv} is the permeability coefficient across the VE, CD is the concentration in the SC, A_{exp} is the exposed surface area, and $P_{sc:ve}$ is the SC:VE partition coefficient. The mass balance differential equation (MBDE) for the SC is

$$\frac{dAD}{dt} = K_{uptake} \times \text{DERMDOSE} - \frac{K_{pv}}{P_{sc:ve}} \times A_{exp} \times CD, \quad [2]$$

where K_{uptake} is the input rate constant and DERMDOSE is the dose to the skin. The rate of input from blood to VE is the cutaneous blood flow rate (QE) times the concentration of naphthalene in blood (CB), and the rate of efflux from the VE to blood is controlled by QE and the solubility of naphthalene in the blood ($P_{ve:b}$). The MBDE for the amount of naphthalene in the VE is

$$\frac{dAE}{dt} = \frac{K_{pv}}{P_{sc:ve}} \times A_{exp} \times CD + QE \times \left(CB - \frac{CE}{P_{ve:b}} \right), \quad [3]$$

where AE is the amount and CE is the concentration of naphthalene in the VE.

Elimination of naphthalene proceeds by two significant mechanisms: exhalation and metabolism. The concentration of naphthalene in end-exhaled air is equal to the blood concentration divided by the blood:air partition coefficient ($P_{b:a}$). Pulmonary clearance of naphthalene is QP divided by $P_{b:a}$. Metabolism of naphthalene occurs in the liver by a single metabolic pathway following first-order kinetics. The initial step in naphthalene metabolism is the formation of naphthalene-1,2-oxide by cytochrome P450 monooxygenases [Agency for Toxic Substances and Disease Registry (ATSDR) 1995]. Liver clearance (Cl_L) is

$$Cl_L = QL \times \left(\frac{V_{max} / K_m}{V_{max} / K_m + QL} \right), \quad [4]$$

where V_{max} (milligrams per minute) is the maximum rate of metabolism, K_m (milligrams per liter) is the Michaelis-Menten constant, and QL the blood flow rate to the liver (liters per minute). The ratio of liver clearance to liver blood flow is the extraction ratio (E_L) where E_L is

$$E_L = \frac{V_{max} / K_m}{V_{max} / K_m + QL}. \quad [5]$$

Determination of the blood:air partition coefficient. Initial sensitivity analysis revealed that the concentration of naphthalene in end-exhaled air was highly sensitive to $P_{b:a}$. Therefore, we measured $P_{b:a}$ by equilibrating human blood with a known concentration of naphthalene (Gargas et al. 1989). Samples were analyzed with a Combi Pal autosampler configured for headspace analysis (CTC Analytics, Zwingen, Switzerland). A series of 20-mL crimp seal vials (MicroLiter Analysis Supplies, Suwanee, GA, USA) containing blood (test), air (reference), and a known amount of naphthalene (gas) were used in the experiment. The test and reference vials underwent the same process. First, the vial was heated to a temperature of 37°C, and a vent tool (LEAP Technologies, Carrboro, NC, USA) was used to equilibrate the pressure between the test/reference vials and the room. Next, a 2.5-mL gas-tight syringe was used to draw from the vial 1 mL of air, which was injected into the room air. Then, 1 mL of gas from the gas vial was transferred to the test/reference vial. The test/reference vial was kept at 37°C and agitated for 1 hr; we determined, by adjusting the incubation period, that 1 hr was the optimal time for achieving equilibrium. After incubation, a 1-mL sample was extracted from the test/reference vial and injected into the GC-MS for analysis. All analyses were conducted in triplicate. A six-point standard curve ($R^2 = 0.999$) was used

for quantitation of the naphthalene concentration in the test and reference vials. Partition coefficients were determined using Equation 6 (Gargas et al. 1989).

$$P_{b:a} = \frac{C_{ref} \times V_{vial} - C_{blood} \times (V_{vial} - V_{blood})}{C_{blood} \times V_{blood}}, \quad [6]$$

where C_{ref} is the naphthalene concentration in the reference vial, V_{vial} is the volume of the reference vial (20 mL), C_{blood} is the naphthalene concentration in the headspace of the test vial, and V_{blood} is the volume of blood (2 mL). Using Equation 6, we calculated a $P_{b:a}$ value of 10.3.

Model optimization. All physiologic parameters (cardiac output, ventilation rate, blood flow rate to the tissues, and tissue volumes) for humans were obtained from the literature (Brown et al. 1997). Other tissue partition coefficients were predicted from the octanol-water partition coefficients and regression models for different tissues (Abraham et al. 1985; Fiserova-Bergerova et al. 1984; Hansch et al. 1995; Willems et al. 2001). The maximum rates of naphthalene metabolism (V_{max}) and Michaelis-Menten constant (K_m) have been estimated for rats and mice (Willems et al. 2001). In our study, the rate of metabolism was assumed to follow first-order kinetics, given the relatively low naphthalene concentrations measured in post-exposure breath samples (Egghy et al. 2003). Initial sensitivity analysis revealed that the concentration of naphthalene in end-exhaled breath was not sensitive to V_{max}/K_m . Therefore, the parameters K_{uptake} , K_{pv} , $P_{f:b}$, and $P_{o:b}$ (other tissue:blood partition coefficient) were adjusted to fit the blood time course data for each volunteer in the laboratory study. Initial values of all parameters were obtained from the literature (Guy and Potts 1992; McCarley and Bunge 2001; Qiao et al. 2000; Willems et al. 2001; Williams and Riviere 1995). The Nelder-Mead algorithm, with tolerance set at 1×10^{-5} , was used to optimize the parameters (Xcellon 2004).

Comparison of dermal and inhalation routes of exposure. The Air Force data set was used to compare the relative contribution of dermal exposure with the end-exhaled breath concentration of naphthalene. The data set included personnel from the U.S. Air Force who had both dermal and inhalation exposures to JP-8 (Chao et al. 2005; Egghy et al. 2003). From the Air Force personnel, end-exhaled breath samples were collected immediately at the end of the work shift and, later, at a central testing site (CTS). Three Air Force personnel were selected who represented the 10th, 50th, and 90th percentiles based on their end-exhaled breath measurements. The group had regular contact with jet fuel, and consisted of fuel-cell maintenance

workers who entered fuel tanks during their work. Thus, the concentration of naphthalene in the air was much higher in the immediate work environment for these individuals compared with personnel who had no direct contact with JP-8 and, therefore, represented background exposures to naphthalene. The air concentration of naphthalene was reported for the duration of the sampling period, which included travel time to the CTS (~ 30 min). Thus, the air concentration at the work site was estimated as

$$INHAL1_{est} = \frac{C_{PBZ} \times \Delta t_{total} - INHAL2_{est} \times \Delta t_{travel}}{\Delta t_{work}}, [7]$$

where $INHAL1_{est}$ is the estimated concentration of naphthalene in the breathing zone during the work shift of Δt_{work} hr, C_{PBZ} is the air concentration measured during the full sampling period of Δt_{total} hr, $INHAL2_{est}$ is the estimated background air concentration of naphthalene (i.e., air measurements from the U.S. Air Force personnel who had no direct contact with JP-8), and Δt_{travel} is the time required to travel from the workplace to the CTS. The concentrations of naphthalene in the air and dermal samples and the duration of exposure were used as input terms for the PBTK model. Predicted end-exhaled breath concentrations were compared with measured levels of naphthalene in end-exhaled breath.

Sensitivity analysis. Sensitivity analyses were performed to evaluate the relative importance of model parameters on the concentration of naphthalene in end-exhaled breath. Normalized sensitivity coefficients (NSC) were calculated using Equation 8 (Evans and Andersen 2000):

$$NCS = \frac{\Delta m}{m} \times \frac{p}{\Delta p}, [8]$$

where m is the response variable (i.e., concentration of naphthalene in end-exhaled breath), Δm is the change in the response variable, p is the value of the parameter of interest (e.g., blood:air partition coefficient), and Δp is the change in the parameter value. Each parameter was changed 1% (i.e., $\Delta p \div p = 0.01$).

Results

Dermal exposure toxicokinetics. The PBTK model was optimized for dermal exposure using data from 10 individuals who were exposed to JP-8 on the skin under laboratory conditions. The average height and weight of the subjects to whom JP-8 was administered on the skin was 174 cm and 61 kg, respectively (BMI = 21 kg/m²). Time-course plots showed considerable variability among the study volunteers (Figure 2). The mean ± SD of the peak concentration of naphthalene in blood was 0.18 ± 0.22 ng/mL and occurred at

62 ± 16 min. The time course for subject no. 1 was very different from that of the other volunteers. The peak concentration for this volunteer was 0.80 ng/mL and occurred at 37 min. Model predictions of the blood concentration of naphthalene are also shown for each volunteer using optimized parameter values in Figure 2. The skin parameters (K_{uptake} and K_{pv}) and the partition coefficients $P_{f:b}$

and $P_{o:b}$ were adjusted to fit the blood time-course data for dermal exposures only; the optimal values are reported in Table 1. The rate of input from dermal exposure is equivalent to the product of the permeability coefficient for the SC (K_{ps}), the exposed surface area (A_{exp}), and the concentration of the naphthalene in JP-8 (C_{JP-8}) (McCarley and Bunge 2001; McDougal and Boeniger 2002):

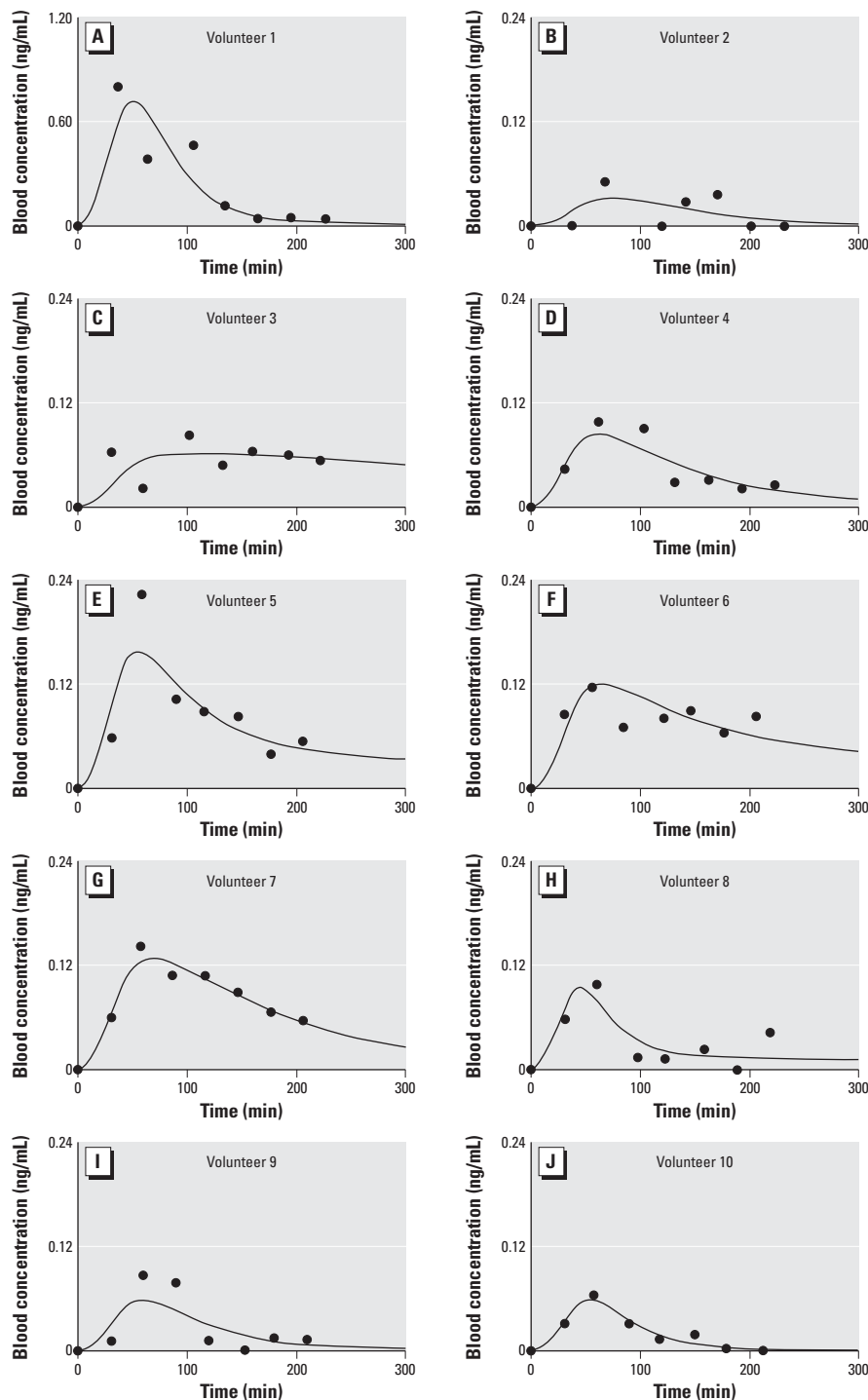


Figure 2. Plots comparing the PBTK model simulations to experimentally measured naphthalene concentrations in blood from 10 study volunteers (A–J) who were dermally exposed to JP-8 on the volar forearm.

$$\begin{aligned} \text{rate of input} &= K_{\text{uptake}} \times \text{DERMDOSE} \\ &= K_{\text{ps}} \times A_{\text{exp}} \times C_{\text{JP-8}} \end{aligned} \quad [9]$$

Equation 9 can be rearranged to solve for K_{ps} as follows:

$$K_{\text{ps}} = \frac{K_{\text{uptake}} \times \text{DERMDOSE}}{A_{\text{exp}} \times C_{\text{JP-8}}} \quad [10]$$

The optimized value of K_{uptake} is $0.031 \pm 0.056 \text{ hr}^{-1}$ (mean \pm SD), and for K_{ps} it is $6.8 \times 10^{-5} \pm 5.8 \times 10^{-5} \text{ cm/hr}$ (mean \pm SD) (Table 2). The sensitive parameters in the dermal only model were *DERMDOSE* (NSC = 1.0), A_{exp} (NSC = 1.0), K_{uptake} (NSC = 1.0), and $P_{\text{o,b}}$ (NSC = -0.3).

Prediction of end-exhaled breath concentrations. The optimized PBTK model was used to predict the end-exhaled breath concentration of naphthalene for 53 U.S. Air Force personnel (13 females and 40 males) who did not have dermal contact with jet fuel and had naphthalene end-exhaled breath concentrations $> 0.0 \mu\text{g}/\text{m}^3$. The median height and weight of the personnel were 175 cm (range, 155–193 cm) and 77 kg (range, 52–116 kg), respectively. In the simulation, the median air concentration of naphthalene was $2.4 \mu\text{g}/\text{m}^3$ (range, 0.7–481.7 $\mu\text{g}/\text{m}^3$) and was held constant for the duration of exposure (median duration, 237 min). For each U.S. Air Force subject, the preexposure concentration of naphthalene in the end-exhaled breath was subtracted from the postexposure measurements. The predicted concentration of naphthalene in end-exhaled breath ($0.5 \mu\text{g}/\text{m}^3$) was the same as the median of the measured values. In addition, comparisons were made between measured and predicted concentrations of naphthalene in end-exhaled breath for each U.S. Air Force subject, using information on the subject's height, weight, air concentration of naphthalene, and duration of exposure. The median relative difference between measured and predicted values was 26% (10th–90th percentile range, -71 to 196%).

Model predictions of the end-exhaled breath concentration of naphthalene were compared with field measurements among three Air Force personnel who represented the 10th, 50th, and 90th percentiles based on their end-exhaled breath measurements. These three U.S. Air Force personnel spent time in a fuel tank during their work shift and were exposed to JP-8 on the skin. The input parameters and values for each U.S. Air Force personnel are reported in Table 3. The PBTK model consistently overpredicted the end-exhaled breath concentrations at the end of work shift for all three U.S. Air Force personnel (Figure 3). This could be attributed to the use of supply-air respirators. Therefore, the air concentration of naphthalene during work (i.e., $\text{INHALI}_{\text{es}}$) was adjusted (i.e., $\text{INHALI}_{\text{adj}}$) to estimate the

likely inhalation exposure (Figure 3). The values of $\text{INHALI}_{\text{adj}}$ are reported in Table 4.

Comparison of dermal and inhalation exposure routes. Simulations were conducted for three U.S. Air Force personnel to compare the contribution of dermal exposure with the end-exhaled breath concentrations relative to inhalation exposure (Table 4). These three individuals were fuel-cell maintenance workers. The area under the end-exhaled breath concentration time curve (AUC_{ex}) was calculated for dermal exposures using the following equation:

$$AUC_{\text{ex}} = \int_{t_0}^{t_1} C_{\text{ex}}(t) dt, \quad [11]$$

where C_{ex} is the concentration of naphthalene in the end-exhaled breath and t_1 is the time at the end of the exposure. The values of AUC_{ex} for dermal exposures were 1.7, 41.7, and $521 \mu\text{g} \times \text{min}/\text{m}^3$ for the 10th, 50th, and 90th percentiles, respectively. Dermal exposures were set to zero and the naphthalene air concentration was adjusted to obtain the same value of AUC_{ex} . The predicted air concentrations ($\text{INHALI}_{\text{pred}}$) were 0.1, 0.7, and $11.7 \mu\text{g}/\text{m}^3$, respectively. These values are 1, 4, and 11% of the air concentrations of naphthalene for the individuals whose breath measurements represented the 10th, 50th, and 90th percentiles, respectively.

Table 1. Naphthalene PBTK model parameters.

Parameter	Symbol	Unit	Value	Notes and references
Body weight	<i>BW</i>	kg	61	Kim et al. (2006a)
Height	<i>HT</i>	cm	174	Kim et al. (2006a)
Body mass index	<i>BMI</i>	kg/m ²	20	<i>BMI = BW/HT²</i>
Organ volumes				
Blood ^a	<i>V_b</i>	L	4.5	<i>BW = (72.447/1000) × BW^{1.007}</i>
Stratum corneum	<i>V_{sc}</i>	L	2×10^{-5}	<i>VD = A_{exp} × Td</i>
Viable epidermis ^b	<i>V_{ve}</i>	L	1.9×10^{-3}	<i>VE = VEC × BW - VD</i>
Fat ^c	<i>V_f</i>	L	5.5	<i>VF = BW × (ln BMI - 126.2)/100</i>
Other tissue	<i>V_o</i>	L	51.0	<i>VO = BW - (VB + VD + VE + VF)</i>
Pulmonary ventilation rate	<i>QP</i>	L/hr/ <i>BW</i> ^{0.75}	15	Brown et al. (1997)
Cardiac output	<i>QC</i>	L/hr/ <i>BW</i> ^{0.75}	15	Brown et al. (1997)
Regional blood flow				
To skin ^d	<i>QE</i>	L/hr	1.7×10^{-2}	<i>QE = QEC × (A_{exp}/SURFA)</i>
To fat	<i>QF</i>	L/hr	16.4	<i>QF = QFC × QC</i>
To other tissues	<i>QO</i>	L/hr	311.0	<i>QO = QC - (QE + QF)</i>
Metabolic clearance parameters				
Ratio of <i>V_{max}:K_m</i>	<i>V_{max}/K_m</i>	L/hr	698	Willems et al. (2001)
Blood flow to liver	<i>QL</i>	L/hr	75.3	<i>QL = QLC × QC</i>
Partition coefficients				
Blood:air	<i>P_{b:a}</i>	—	10.3	Measured ^e
Stratum corneum:viable epidermis	<i>P_{sc:ve}</i>	—	1.8	McCarley and Bunge (2001)
VE:blood	<i>P_{ve:b}</i>	—	2.8	McCarley and Bunge (2001)
Fat:blood	<i>P_{f:b}</i>	—	25.6	Estimated ^f
Other tissue:blood	<i>P_{o:b}</i>	—	5.2	Estimated ^f
Skin permeation parameters				
Area of exposure	<i>A_{exp}</i>	cm ²	20	Dimensions of the tape strip
Thickness of the stratum corneum	<i>Td</i>	μm	10	McCarley and Bunge (2001)
Total body surface area ^g	<i>SURFA</i>	cm ²	19,238	<i>(BW^{0.45}) × (HT^{0.725}) × 71.84</i>
Permeability coefficient for stratum corneum	<i>K_{ps}</i>	cm/hr	6.8×10^{-5}	Estimated ^f
Permeability coefficient for viable epidermis	<i>K_{pv}</i>	cm/hr	3.0×10^{-3}	Estimated ^f

^aFrom Davies and Morris 1993. ^bThe volume of the viable epidermis is calculated as the volume of the exposed skin minus the volume of the stratum corneum under the exposed area. The fraction of body weight in skin (VEC) is from Brown et al. (1997). ^cThe fraction of body weight in fat = $\ln \text{BMI} - 126.2$ (Mills 2005). ^dThe fractions of cardiac output to skin (QEC) and to liver (QLC) were obtained from Brown et al. (1997). ^eThe blood:air partition coefficient was measured using the vial equilibration technique (Gargas et al. 1989). ^fModel parameters were estimated by fitting the model to the data (Figure 2). ^gTotal body surface area (Haycock et al. 1978).

Table 2. Optimized values of the skin parameters K_{uptake} , K_{pv} , $P_{\text{f:b}}$, and $P_{\text{o:b}}$. K_{ps} were calculated using Equation 10. The parameters were optimized for each of the 10 study volunteers.

Volunteer	$K_{\text{uptake}} \times 10^{-3} (\text{hr}^{-1})$	$K_{\text{ps}} \times 10^{-5} (\text{cm/hr})$	$K_{\text{pv}} \times 10^{-3} (\text{cm/hr})$	$P_{\text{f:b}}$	$P_{\text{o:b}}$
1	190.7	18.8	7.6	4.4	0.6
2	4.4	1.1	1.5	0.1	0.6
3	13.6	8.0	0.6	1.6	8.9
4	21.3	3.2	3.1	15.2	2.4
5	16.9	11.8	2.4	15.4	12.2
6	22.8	11.7	1.7	3.1	8.3
7	12.2	6.8	2.1	22.4	2.7
8	18.6	4.1	5.4	11.1	16.1
9	8.3	1.5	2.2	7.3	0.3
10	3.7	1.3	3.5	175.7	0.1
Mean \pm SD	31.3 ± 56.4	6.8 ± 5.8	3.0 ± 2.1	25.6 ± 53.2	5.2 ± 5.8

Sensitivity analysis. Normalized sensitivity coefficients (mean) were calculated separately for exposure and physiologic parameters. The sensitivity analysis was conducted for a typical mixed exposure scenario, that is, the subject representing the 50th percentile in terms of end-exhaled breath measurements. Each parameter was changed 1% from its optimal value (Table 1) in the forward direction (Equation 8). The response variable in both sets of calculations was the concentration of naphthalene in the end-exhaled breath. For exposure variables, the end-exhaled breath concentration was most sensitive to the estimated air concentration of naphthalene during work ($NSC = 1.0$). End-exhaled breath concentrations were not sensitive to the variables $DERMDOSE$ and A_{exp} , as the dermal route accounts for only a small percentage of total exposure in these individuals. In the multidose route PBTK model, the end-exhaled breath concentration of naphthalene was most sensitive to cardiac output ($NSC = -0.7$), ventilation rate ($NSC = 0.9$), and the blood:air partition coefficient ($NSC = -0.9$) (Figure 4). The $NSCs$ for other parameters were < 10.21 .

Discussion

A PBTK model was developed to predict end-exhaled breath concentrations of naphthalene from dermal and inhalation exposure to JP-8. Our model consisted of five compartments representing the stratum corneum, viable epidermis, blood, fat, and other tissues, and contains fewer parameters than previously published physiologically based compartmental models of naphthalene (Quick and Shuler 1999; Willems

et al. 2001). The fat was considered separate from the other tissues because the time constant for fat (8.6 hr) was larger than the time constant for other tissues (0.9 hr). However, the other tissue compartment was included in the model because the skin compartment consisted of the skin directly under the exposed area. The remaining skin was included in the other tissue compartment.

Adjustments were made to the fat:blood and other tissue:blood partition coefficients for the PBTK model predictions to fit the experimental and occupational exposure data. For many chemicals, the partition coefficients are not known. In such cases, quantitative structure–activity relationship (QSAR) models may be used to predict the necessary partition coefficients; however, the predictions are limited to chemicals with physicochemical properties that lie within the calibration data set (Beliveau et al. 2003). In our study we calibrated the values of P_{fb} and $P_{o,b}$, which were predicted by Willems et al. (2001) using QSAR models, against human exposure data. We estimated a P_{fb} value of 25.6 for naphthalene, which is more plausible than 160 given that the P_{fb} for benzene is 55 and 25 for decane. Using the vial equilibration technique of Gargas et al. (1989), we also measured a $P_{b,a}$ value of 10.3 for naphthalene, which is more consistent with the $P_{b,a}$ for other compounds than is the value of 571 reported by Willems et al. (2001). For example, the human $P_{b,a}$ for benzene, cyclohexane, JP-10, and *p*-xylene were 8.19, 1.41, 52.5, and 44.7, respectively (Gargas et al. 1989).

The PBTK model was used to calculate the permeability coefficient (K_p) for naphthalene in humans *in vivo*. Previously, the K_p had been

calculated using Fick's law of diffusion. A K_p value of 5.1×10^{-4} cm/hr was estimated for rat skin *in vitro* (McDougal et al. 2000). This *in vitro* K_p value was compared with a K_p value that was estimated by calculating the flux value for aromatic and aliphatic components of JP-8 in humans from the slope of the linear portion of the cumulative mass of chemical in blood per square centimeter versus time curve (Kim et al. 2006a). We calculated an apparent K_p of 5.3×10^{-5} cm/hr, which is approximately an order of magnitude lower than that for the rat K_p . This K_p calculation was revised using a DTK model and Equation 9. A larger K_p value was estimated (1.8×10^{-3} cm/hr), which was more similar to the K_p estimated *in vitro* by McDougal et al. (2000). The limitation of using a data-based compartmental model is that the parameter values are not constrained by the actual anatomy and physiology of the human body and the biochemistry of naphthalene *in vivo*. We incorporated such constraints into our PBTK model and revised our calculation of K_p for naphthalene. We estimated a K_{ps} value of 6.8×10^{-5} cm/hr and a K_{pv} value of 3.0×10^{-3} cm/hr. The value of K_{eff} , which is the overall permeability coefficient for chemicals crossing the skin (McCarley and Bunge 2001), is 6.6×10^{-5} cm/hr.

K_{eff} is approximately 7-fold smaller than the K_p reported by McDougal et al. (2000). A 7-fold difference was not unexpected because rat skin used in the McDougal et al. (2000) study is generally considered more permeable than human skin. Molecular diffusion is the dominant mechanism that governs the permeation of naphthalene across the skin. For diffusion, the flux (and K_p) is inversely proportional to the thickness of the diffusion distance, as stated by Fick's first law of diffusion. Therefore, doubling the thickness of the skin will result in halving the K_p . McDougal et al. (2000) estimated K_p across rat skin of thickness 560 μ m. The human skin thickness ranges from 500 μ m to 4,000 μ m; therefore, the human K_p value is expected to be between 6.4×10^{-5} cm/hr and 5.7×10^{-4} cm/hr. Our estimate of the effective permeability coefficient for naphthalene lies within this range of expected values.

Table 3. Input parameters and values for prediction of end-exhaled breath concentrations of naphthalene in the U.S. Air Force personnel who represented the 10th, 50th, and 90th percentiles based on end-exhaled breath measurements.

Variable	Percentile		
	10th	50th	90th
Height (cm)	175	188	168
Body weight (kg)	81	109	73
$INHAL1_{est}$ (μ g/m ³)	499	322	3,640
$INHAL2_{est}$ (μ g/m ³)	2.0	2.0	2.0
$DERMDOSE$ (μ g/cm ²)	3.9×10^{-5}	5.5×10^{-4}	9.2×10^{-3}
Duration of exposure (min)	224	322	260

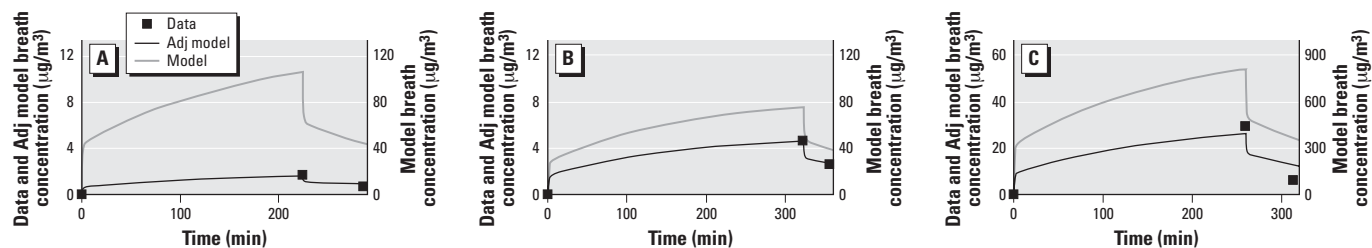


Figure 3. Model simulations and end-exhaled breath concentrations for the U.S. Air Force personnel who were exposed to JP-8 via inhalation and dermal routes. Breath samples were collected immediately at the end of the work shift and at a central testing site. Shown are the measured and predicted values for three U.S. Air Force personnel who represented the 10th (A), 50th (B), and 90th (C) percentiles of measured end-exhaled breath concentrations. Simulations are also shown after adjusting the air concentration of naphthalene during work to better estimate the true inhalation exposure [adjusted (Adj) model].

The optimized PBTK model was used to predict end-exhaled breath measurements collected in the workplace for the U.S. Air Force personnel exposed to JP-8 by the inhalation route. The predicted concentration at the end of their work shift was the same as the measured values. Further comparisons of predicted versus measured values revealed considerable interindividual variability. Sources of heterogeneity in a population may include physical condition, level of activity, disease state, age, hormonal status, and interactions with other chemicals and drugs (Clewell and Andersen 1996). Further, we observed considerable variation in the values of K_{ps} and K_{pv} , but the small sample size (10 subjects) limited the analysis of variability in our study. Further study of the heterogeneity of parameter values and the impact on the toxicokinetic profile of naphthalene in humans is needed.

We also used the optimized PBTK model to examine dermal and inhalation exposure to JP-8. Three U.S. Air Force personnel were selected who represented the 10th, 50th, and 90th percentiles based on their end-exhaled breath concentrations. The predicted concentrations of naphthalene were well above what was expected in end-exhaled breath. For example, for the personnel representing the

50th percentile, $INHAL1_{est}$ overpredicted the end-exhaled breath concentration of naphthalene by 1,540% (i.e., 75.8 $\mu\text{g}/\text{m}^3$ vs. 4.6 $\mu\text{g}/\text{m}^3$). The reason for overpredicting breath concentrations was that these workers wore personal protective equipment that included forced supply-air respirators while working in fuel tanks. Thus, the air concentration that was measured using the passive monitors was not the actual air concentration that the Air Force personnel were exposed to while working inside the fuel tanks.

The PBTK model was exercised to obtain a better estimate of the air concentration that corresponded to the breath measurements. The adjusted air concentration was used in our calculation of the relative contribution of dermal exposure to the end-exhaled breath concentration of naphthalene. We observed that the median contribution of dermal exposure to the end-exhaled breath concentration of naphthalene was relatively small (4%). However, in the U.S. Air Force personnel who represented the 90th percentile, the relative contribution of dermal exposure to the end-exhaled breath concentration was 11%. The U.S. Air Force personnel examined in this study comprised fuel-cell maintenance workers. Thus, the use of dermal protective equipment can further

decrease the end-exhaled breath concentration of naphthalene in the fuel-cell maintenance workers.

This PBTK model has reduced the uncertainty in modeling JP-8 exposures because fewer parameters were required to predict the time-course of naphthalene. However, our model has identified some data gaps. First, inhalation exposures should be measured over shorter time intervals. Sensitivity analysis demonstrated that end-exhaled breath levels of naphthalene were most sensitive to the air concentration of naphthalene during work. In our study, we used time-weighted average concentrations (over approximately 4 hr) that did not capture exposures to high levels of naphthalene from local sources. Therefore, shorter time-resolved data may be used to better explain the transient nature of inhalation exposures to JP-8. Second, occupational and environmental exposure studies of other components of JP-8 are needed to gain a more complete picture of JP-8 exposures. Currently, occupational exposure studies have focused on single chemical components of JP-8. The results of multichemical exposure assessment studies may be compared with results from single-chemical studies and add to our understanding of the absorption, distribution, metabolism, and elimination of complex chemical mixtures.

The modeling approach used in this study represents a useful technique for assessing the contribution of dermal and inhalation exposures to the systemic levels of chemicals. One of the primary applications of this work could be to improve the understanding of exposure processes by quantifying the relationship between external exposure measurements and biomarkers of internal dose. For example, a series of air and dermal exposure measurements may be collected from a sample of individuals from groups stratified by fixed factors such as location relative to the source of exposure. One could, for example, introduce an intervention (e.g., respirators) and use the PBTK model to quantify the efficacy of the intervention for reducing systemic levels of the toxicant. This approach would be useful for protecting the health of individuals. For example, if the concentration of the exposure biomarker (i.e., blood and/or breath concentration) is driven primarily by the dermal route in a given group, there would be little advantage in additional respiratory protection. This approach may be used in both occupational and environmental risk assessment applications. However, additional modeling and experimental studies are required before generalization of this model to confirm scenarios/dose metrics beyond the limitations of the current study.

In conclusion, we used the PBTK model to quantify the contribution of dermal exposures

Table 4. Estimated contribution of dermal exposure to the end-exhaled breath concentrations of naphthalene relative to inhalation exposure.^a

Percentile	Breath ($\mu\text{g}/\text{m}^3$)	AUC_{ex} ($\mu\text{g} \times \text{min}/\text{m}^3$)	$INHAL1_{adj}$ ($\mu\text{g}/\text{m}^3$)	$INHAL1_{pred}$ ($\mu\text{g}/\text{m}^3$)	Ratio (%)
10th	1.7	1.7	7.4	0.1	1
50th	4.7	41.7	18.8	0.7	4
90th	29.4	521	103	11.7	11

^aThis analysis was based on three U.S. Air Force personnel whose end-exhaled breath concentrations represented the 10th, 50th, and 90th percentiles. The ratio of $INHAL1_{pred}$ to $INHAL1_{adj}$ is a measure of the relative percent contribution of dermal exposure to the end-exhaled breath concentration.

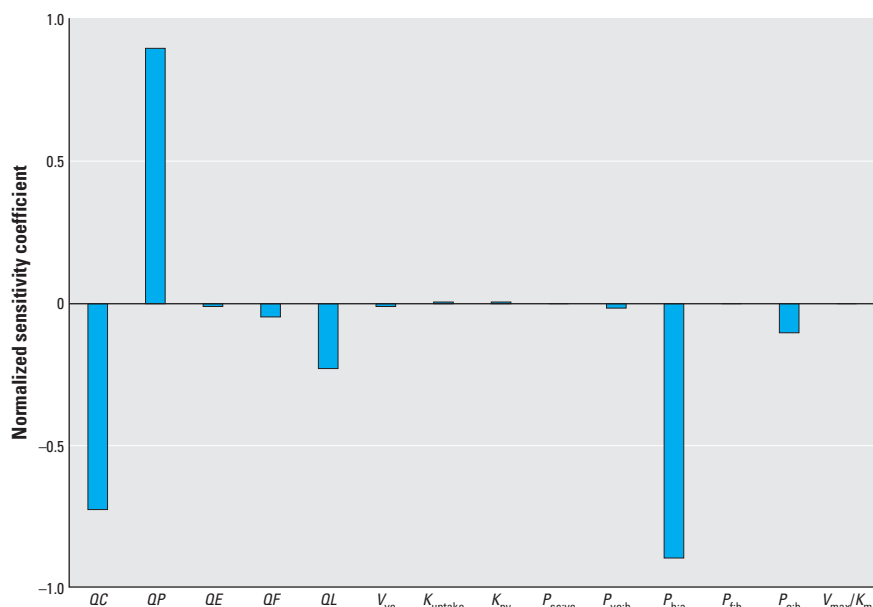


Figure 4. Normalized sensitivity coefficients for the end-exhaled breath concentrations. Parameters were adjusted at the 1% level.

to the systemic levels of naphthalene. We estimated a permeability coefficient that was 7-fold lower than estimates for rat skin made *in vitro*. Our approach used a combination of exposure assessment, biological monitoring, and toxicokinetic modeling tools to integrate external exposure and biomarker data into a single description of the toxicokinetic behavior of naphthalene. The PBTK model incorporated exposures from both dermal and inhalation routes and required estimation of fewer parameters than previously published PBTK models of naphthalene. This PBTK model, which included two major exposure routes relevant to occupational and environmental exposure scenarios, may be used for integrating animal and human observational studies into an improved understanding of human health risks for JP-8. A wide range of permeability coefficient values was noted in the individuals in this research and further study of the sources of inter- and intraindividual variation in these processes appears necessary.

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Estradiol and Bisphenol A Stimulate Androgen Receptor and Estrogen Receptor Gene Expression in Fetal Mouse Prostate Mesenchyme Cells

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BACKGROUND: Hormonal alterations during development have lifelong effects on the prostate gland. Endogenous estrogens, including 17 β -estradiol (E₂), and synthetic estrogenic endocrine disruptors, such as bisphenol A (BPA), have similar effects on prostate development. Increasing exposure to estrogens within the low-dose, physiologic range results in permanent increases in the size and androgen responsiveness of the prostate, whereas exposure within the high-dose, pharmacologic range has the opposite effects.

OBJECTIVES: We tested the hypothesis that the low-dose effects of estrogens on the developing prostate are associated with increased expression of androgen receptor (*Ar*) and estrogen receptor 1 (α) (*Esr1*) genes in mesenchyme cells.

METHODS: *Ar* and *Esr1* mRNA levels were quantified in primary cultures of fetal mouse prostate mesenchyme cells treated with E₂ and BPA.

DISCUSSION: *Ar* and *Esr1* mRNA expression increased in response to E₂, with thresholds of 0.001 and 0.037 nM, respectively; and in response to BPA, with a threshold of 1 nM for both mRNAs. We did not observe the expected inhibition of *Ar* mRNA expression by pharmacologic levels of E₂ relative to unexposed cells.

CONCLUSIONS: The observed induction of gene expression occurred at concentrations within the range of free E₂ previously shown to permanently increase prostate size, thus supporting the involvement of direct effects of estrogens on gene expression in prostate mesenchyme. The effects of BPA occurred within the range of concentrations currently measured in human serum, demonstrating the vulnerability of developing tissues to xenoestrogens.

KEY WORDS: 17 β -estradiol, androgen receptor gene, bisphenol A, dose-response relationship, estrogen receptor 1 (α) gene, prostate, sexual differentiation. *Environ Health Perspect* 115:902-908 (2007). doi:10.1289/ehp.9804 available via <http://dx.doi.org/> [Online 27 February 2007]

During fetal life, alterations in normal prostate gland development can produce permanent changes that persist throughout adulthood and may increase the risk of disease in later life (Ho et al. 2006; Risbridger et al. 2005). The prostate differentiates from the cranial region of the urogenital sinus (UGS) (Marker et al. 2003). In humans, the first epithelial buds are observed in the lateral region of the UGS during the tenth week of gestation in a pattern that shows a remarkable similarity to that of bud development in mice and rats during the early phase of gland genesis (Timms et al. 1994). Prostate ductal budding begins on gestation day (GD) 17 in mice (2 days before birth) (Timms et al. 1994). Prostate development is dependent on 5 α -dihydrotestosterone (DHT) production from testosterone within the UGS mesenchyme (Marker et al. 2003). Androgen receptor expression in prostatic mesenchyme is required for directing growth and branching morphogenesis of epithelial buds, presumably by induction of paracrine factors secreted by mesenchyme (Cunha and Donjacour 1987; Kokontis and Liao 1999). During development, prostatic epithelial cells exhibit little androgen binding, and androgen receptor protein expression in epithelium is not required for differentiation (Cunha and Donjacour 1987; Prins and Birch 1995; Timms et al. 1999). Therefore,

fetal mouse UGS mesenchyme cells provide an informative model of endocrine control of prostate development.

There is now considerable evidence that estrogens modulate the activity of androgens in regulating prostate development. The UGS mesenchyme in mice and rats responds to estrogens via estrogen receptor 1 (α), whereas in the human prostate estrogen receptor 2 (β) may mediate most responses to estrogens during development (Adams et al. 2002; Prins et al. 1998). Prostatic growth and androgen receptor ligand-binding activity are permanently decreased in response to high, pharmacologic doses of both natural and xenobiotic estrogens during development (Prins and Birch 1995; Rajfer and Coffey 1978; vom Saal et al. 1997). In contrast, increases in prenatal estrogen levels within the physiologic range (the normal range for endogenous estradiol) stimulate prostate development, leading to permanently increased prostate size and androgen receptor ligand-binding activity (Gupta 2000; Timms et al. 1999; vom Saal et al. 1997).

Estrogenic endocrine disruptors have the potential to alter prostate development in a manner similar to that of endogenous estradiol. In this study, we chose to examine bisphenol A (BPA), the monomer used to make polycarbonate plastic and as an additive in many other plastic products. BPA is

produced in excess of 6 billion pounds per year, and the potential for human exposure is great due to leaching from plastic and plastic-lined metal food and beverage containers, as well as from dental sealants (Takao et al. 2002; Welshons et al. 2006).

We have proposed that one mechanism by which fetal estrogen exposure stimulates prostate development is by increasing prostatic androgen receptor gene [*Ar*; GenBank accession no. X53779 (Benson et al. 2007)] expression, thereby increasing the androgen responsiveness of the developing prostate, leading to enhanced gland genesis and growth (Richter et al. 2005; vom Saal et al. 1997). In the present study we sought to determine whether the endogenous hormone 17 β -estradiol (E₂), within its physiologic range, and the manmade estrogenic endocrine disruptor BPA, within the range measured in human serum (Schönfelder et al. 2002), directly influence *Ar* and estrogen receptor 1 (α) (*Esr1*; GenBank accession no. NM_007956.2) gene expression at the transcriptional level in fetal mouse UGS mesenchyme.

Materials and Methods

Animals, housing, mating, and organ collection. CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and bred at the University of Missouri in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Animals were housed on corn-cob bedding in standard polypropylene cages. They received water purified by ion exchange and carbon filtration from glass bottles. Pregnant and lactating females were fed Purina 5008 chow (Purina Mills, St. Louis, MO). After being weaned, animals were fed

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Support was provided by S. Khan, G.S. Johnson, and D. Lubahn, and by grants ES-11283 (F.S.vS), 1F32ES-11549-01 (C.A.R.), and P01ES10535 (D. Lubahn) from the National Institute of Environmental Health Sciences (NIEHS).

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The authors declare they have no competing financial interests.

Received 5 October 2006; accepted 27 February 2007.

Purina 5001 chow (Ralston Purina). Rooms were maintained at 25 ± 2°C under a 12 hr:12 hr light:dark cycle. Animals were treated humanely and with regard for alleviation of suffering. Animal procedures were approved by the University of Missouri Animal Care and Use Committee and conformed to the NIH *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996).

Tissue collection, primary cell culture, and dosing. Timed-pregnant females were killed on GD17 (mating = GD0) by CO₂ asphyxiation, and fetuses were removed from the uterine horns. The bladder and UGS were removed from male fetuses as previously described (Timms et al. 1999; vom Saal et al. 1997). The prostatic region of the UGS was removed from the bladder at the bladder neck, and mesenchymal cells were isolated as described by Gupta (1999). Briefly, UGS tissue was disrupted by digestion with 3 mg collagenase type I/mL (Sigma Chemical Co., St. Louis, MO) for 30–50 min at 37°C in a shaking water bath followed by manual pipetting. Clumps of epithelium were allowed to settle out, and suspended mesenchymal cells were collected and cultured in complete medium [RPMI-1640 without phenol red (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U penicillin G sodium/mL, 100 mg streptomycin sulfate/mL, and 0.25 mg fungizone/mL] with 10% (vol/vol) fetal bovine serum (FBS; U.S. Bio-Technologies, Parkerford, PA). Cells were grown to 95% confluence and then passaged by digestion with 0.05% trypsin in 0.53 mM EDTA (Gibco) for 5 min at room temperature. Cell viability was assayed with alamarBlue (BioSource International, Camarillo, CA) according to the manufacturer's instructions.

We characterized the cell-type composition of the UGS cell primary cultures by immunofluorescent staining of cytokeratins with mouse anti-pan-cytokeratin clone PCK-26 fluorescein isothiocyanate conjugate (Sigma), and co-staining of the mesenchymal cell marker vimentin with goat anti-vimentin (Sigma) and rabbit anti-goat Cy3 conjugate (Sigma) (Prins et al. 1991).

During experimental treatments with E₂, BPA, tamoxifen, and raloxifene, FBS was charcoal-stripped to remove all hormones, and cells were maintained in a constant background of 690 pM DHT (200 pg/mL). Cells were treated with DHT rather than testosterone to control for potential treatment effects on the intracellular concentration of this high-affinity ligand for the androgen receptor, which is formed from testosterone in UGS mesenchyme cells *in vivo*, and also to avoid the intracellular metabolism of testosterone to E₂ by aromatase; DHT is not a substrate for aromatase (Kokontis and Liao 1999). First passage cells were seeded onto 24-well plates at 7 × 10⁴ cells/well in estrogen-free complete medium with 5% (vol/vol) charcoal-stripped FBS, 5% (vol/vol) charcoal-stripped horse serum (Sigma), 690 pM DHT (Steraloids, Wilton, NH), and ITS supplement (insulin-transferrin-selenium; Cambrex, Walkersville, MD) for final concentrations of 10 µg insulin/mL, 10 µg transferrin/mL, and 10 ng selenium/mL. Cells were maintained in this estrogen-free medium for 3 days, with one medium change, before the start of treatments. E₂, BPA, and tamoxifen were obtained from Sigma. Raloxifene (LY 156,758) was obtained from Eli Lilly (Indianapolis, IN). During treatments with E₂ and BPA, cells were grown for 4 days, and the medium was changed every day, except where noted. The concentration of E₂ in culture medium during treatments was measured by radioimmunoassay as previously described by vom Saal et al. (1990).

Real time RT-PCR measurement of gene expression. Total RNA was isolated with the RNAqueous kit (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNA was quantified by absorbance at 260 nm. Expression of specific mRNAs were measured by one-step real-time reverse transcription-polymerase chain reaction (RT-PCR) as described by Bustin (2000), with the TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA) on the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The concentrations of Mn²⁺,

probe, and primers were optimized for each primer/probe set. Primer/probe sets for *Ar*, vimentin (*Vim*; GenBank accession no. NM_011701.3), and acidic ribosomal phosphoprotein P0 (*Arbp*; GenBank accession no. NM_007475.2) were designed using Primer Express software (PE Applied Biosystems) and are shown in Table 1. Primers were designed to span exon boundaries in order to prevent amplification of genomic DNA. Primers were synthesized by Invitrogen (Carlsbad, CA), and probes were synthesized by PE Applied Biosystems. The primer/probe set for *Esr1* was TaqMan Gene Expression Assay ID Mm00433149_m1 (PE Applied Biosystems), which spans *Esr1* exons 3–4.

The relative concentrations of specific mRNAs in each sample were normalized to total RNA per well, as described by Bustin (2000) and Latil et al. (2001). Normalization to total RNA allowed for comparisons between independent experiments. In parallel experiments, total DNA per well was measured by fluorescence of Hoechst 33258 (Sigma), as described by Labarca and Paigen (1980). From these data, the average RNA/DNA ratio was calculated for each treatment; we used these values to convert the mRNA/total RNA measurements to mRNA/DNA to assess the effect of E₂ and BPA on gene expression per cell.

Statistical analyses. Treatments were replicated in three wells per experiment and at least two, and in most cases more (up to 10), replicate experiments. Outliers were detected with Grubbs' test (Grubbs 1969) and removed. Treatment effects were evaluated on untransformed data for RNA, and on reciprocals for DNA and gene expression, with the analysis of variance (ANOVA) general linear model procedure using SAS software (SAS Institute Inc., Cary, NC). We made planned comparisons of means for each treatment relative to controls using the least-squares means test only if the overall ANOVA showed significant treatment effects. To avoid inflation of error rates, we did not use multiple comparisons among all treatments. The criterion for statistical significance was *p* < 0.05.

Results

Characterization of UGS cells and nominal E₂ concentration in primary culture. Consistent with previous reports (Gupta 1999), immunofluorescent staining for the mesenchymal cell marker vimentin revealed no epithelial cells in first passage cells treated for 5 days with 0.1 nM E₂ or with no E₂ (data not shown). The UGS primary cell cultures that we examined were thus homogenous populations of mesenchyme cells that retained mesenchymal differentiation markers throughout the incubation period.

After the initial administration of E₂ in culture medium, the E₂ concentration slowly

Table 1. Sequences of primers and probes for real time RT-PCR assays.

Gene	Sequence (5' → 3')	5' position in CDS	Exon boundary
<i>Ar</i>			
Forward	TGTCAAAGTGAAATGGGACC	1494	
Reverse	TGGTACTGTCCAAACGCATGT	1567	1–2 at 1553
Probe	TGGATGGAGAAGTACTCCGGACCTTATGGG	1516	
<i>Arbp</i>			
Forward	GAGATTCGGGATATGCTGTTGG	289	2–3 at 302
Reverse	GGCGATGGCACCAGCC	351	
Probe	CAATAAGGTGCCAGCTGCTGCTCG	312	
<i>Vim</i>			
Forward	GCACCCTGCAGTCATTGAGA	602	
Reverse	CCACTTCCGTTCAAGGTCAA	673	3–4 at 660
Probe	AGGATGTTGACAATGCTTCTGGCACG	623	

CDS, coding sequence.

decreased, presumably by metabolism, sequestration in cells, and/or binding to the tissue culture dish. In more detail, E₂ was administered at a concentration of 1 nM, in the middle of the dose range in our experiment. The

concentration of E₂ in medium decreased by 2 hr to approximately 90% and by 18 hr to approximately 60% of the administered dose, and then remained stable through the remaining time period examined (up to 48 hr).

In the experiments we conducted, test chemicals were added to medium every 24 hr. Thus, at the midpoint of the dose–response curve tested, the actual E₂ concentration in the culture medium was approximately 60% of the initial concentration at the time we collected the cells for analysis of mRNA. Measurement of DNA and RNA content and induction of gene expression confirmed that bioactive amounts of E₂ were thus present at nominal concentrations < 0.001 nM (Figures 1 and 2).

E₂ induces growth of UGS mesenchyme cells. E₂ treatment induced a small increase in cell growth and proliferation as indicated by DNA and RNA content at doses of 0.01–10,000 nM (Figure 1A, 1B). At 100,000 nM E₂, inhibition of cell growth and proliferation was observed (Figure 1A, 1B). Cell viability was not affected at any E₂ dose tested (data not shown). Subsequent experiments used a dose range of 0.0001–10,000 nM in order to avoid the cell growth–inhibiting effects of very high doses of E₂. Relative total RNA was induced to a greater degree than DNA (Figure 1A, 1B). The housekeeping genes *Vim*, a component of the cytoskeleton in mesenchyme cells, and *Arbp*, a component of the ribosome, were examined to determine whether either could be used as a reference gene. However, both of these genes increased expression in response to E₂, consistent with a general induction of cell growth (Figure 1C, 1D). Vimentin and acidic ribosomal phosphoprotein P0 exhibited differently shaped dose–response curves, suggesting differences in their mechanisms of transcriptional regulation by E₂. Because neither housekeeping gene was an appropriate control gene, expression of specific mRNAs was normalized to DNA.

E₂ induces the steroid receptor mRNAs *Ar* and *Esr1*. *Ar* mRNA expression was induced by E₂ up to just over 2-fold above control levels (Figure 2A). The observed threshold of induction, 0.001 nM, is slightly higher than the measured free serum E₂ concentration of 0.00077 nM or 0.21 pg/mL in male mouse fetuses on GD18 (vom Saal et al. 1997). The increase in *Ar* mRNA with E₂ dose was monotonic up to 100 nM E₂. At doses of ≥ 1,000 nM E₂, *Ar* mRNA levels declined relative to the maximum observed induction at 100 nM E₂. Inhibition of cell growth was only evident at 100,000 nM E₂.

The induction of *Ar* mRNA by a physiologically relevant level of E₂, 0.037 nM (10 pg/mL), was significantly inhibited by antiestrogen treatment (Figure 3A). The antiestrogen raloxifene (100 nM) had similar effects to 100 nM tamoxifen (raloxifene data not shown). The inhibition of the *Ar* response to E₂ by tamoxifen was overcome by addition of a pharmacologic dose of 100 nM E₂, demonstrating that the inhibition by tamoxifen observed at 0.037 nM E₂ is not due

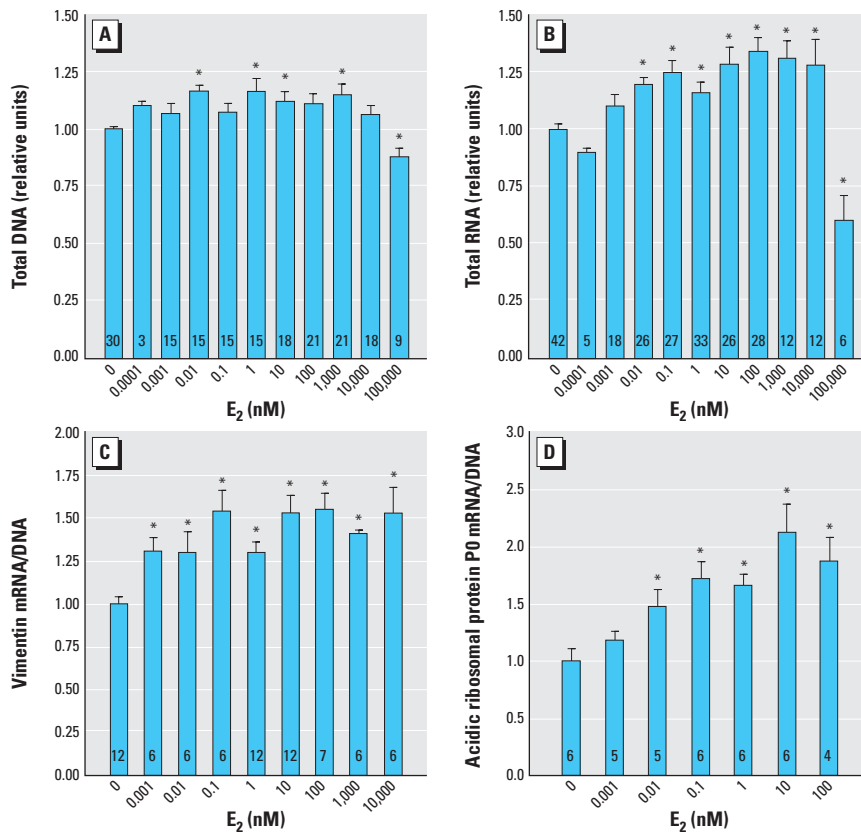


Figure 1. Indicators of cell growth increase with E₂ dose. (A) DNA content slightly increased with E₂ in a dose-independent manner up to 10,000 nM and decreased significantly at 100,000 nM E₂. (B) Total RNA content increased with E₂ in a dose-dependent manner up to 10,000 nM and decreased significantly at 100,000 nM E₂. (C and D) Gene expression of the cytoskeleton protein vimentin (C) and the ribosomal component acidic ribosomal phosphoprotein P0 (D) increased with E₂ treatment. Units are fold induction relative to the control. Error bars represent 1 SE. The number of replicates measured for each treatment is shown in each bar.

*Values significantly different from the control ($p < 0.05$).

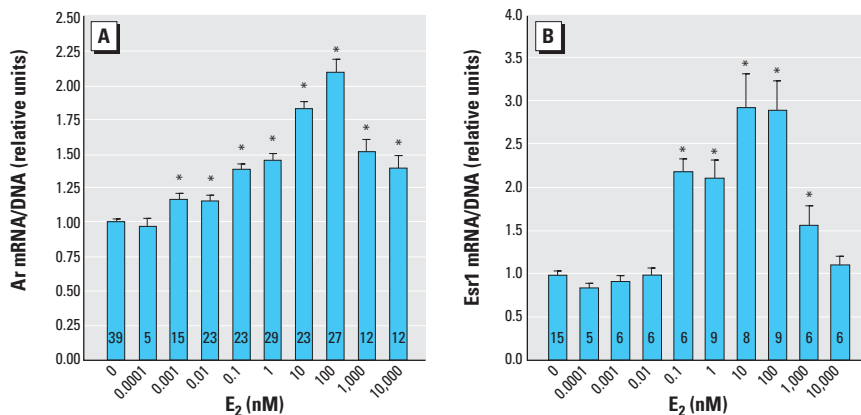


Figure 2. Biphasic *Ar* and *Esr1* gene expression responses induced by E₂. (A) *Ar* mRNA expression increases with increasing dose of E₂ up to a dose of 100 nM. A dose of 0.001 nM (0.27 pg/mL) is within the physiologic range of free E₂ in mouse fetuses, and a significant response to this dose is consistent with prior *in vivo* findings (vom Saal et al. 1997). (B) *Esr1* mRNA expression increases with increasing dose of E₂ up to a dose of 100 nM. Error bars represent 1 SE. The number of replicates measured for each treatment is shown in each bar.

*Values significantly different from the control ($p < 0.05$).

to cytotoxicity or other nonspecific effects (Figure 3A).

Esr1 mRNA expression was induced by E₂ by approximately 3-fold over the control, with a threshold at 0.037 nM (Figure 3B) and a peak at 10–100 nM E₂ (Figure 2B). The induction of *Esr1* mRNA by 0.037 nM E₂ was not significantly inhibited by anti-estrogen treatment (Figure 3B).

BPA acts as an estrogen agonist in UGS mesenchyme cells. The effects of BPA on cell growth as indicated by DNA and RNA content (Figure 4) were much less pronounced than the effects of E₂ (Figure 1). The dose–response curve for RNA was biphasic, with significant reductions in RNA content, but not DNA, at very low concentrations of BPA (Figure 4). *Ar* and *Esr1* mRNA expression were induced by BPA treatment (Figure 5). The dose–response curves for BPA were shifted to the right by approximately 1,000-fold for *Ar* and approximately 30-fold for *Esr1*, relative to E₂ (based on the significant stimulation of *Esr1* in response to 0.037 nM E₂; Figure 3B). As indicated in Figure 5A and 5B, a significant increase in both *Ar* and *Esr1* transcription occurred at BPA concentrations within the range typically reported in human blood and tissues, including fetal blood (Schönfelder et al. 2002; Welshons et al. 2006). The induction of both *Ar* and *Esr1* mRNAs by a physiologically relevant low dose of BPA (10 nM) was inhibited by a 100-nM dose of tamoxifen. For both genes, the inhibition by tamoxifen was overcome by a high dose of BPA (1,000 nM) (Figure 6).

Discussion

The aims of the present study were to investigate whether there were direct effects on *Ar* and *Esr1* gene activity that could be related to the previously observed stimulatory effect on prostate development caused by prenatal exposure to serum concentrations of bioactive E₂ in fetal mice and the concentration of bioactive BPA currently measured in human fetal serum. We found that both the natural estrogen E₂ and the synthetic estrogenic endocrine disruptor BPA stimulated increases in prostate *Ar* and *Esr1* mRNAs. The stimulation occurred at physiologically relevant part-per-trillion doses of E₂, and at parts-per-billion doses of BPA, which are within the range found in human fetal blood (reviewed by Welshons et al. 2006).

Exposure of male mouse and rat fetuses to slightly elevated estrogen levels results in permanent prostate enlargement and elevated androgen receptor levels in adulthood (Timms et al. 1999; vom Saal et al. 1997). Some confusion concerning effects of estrogen on the prostate has been created by studies in which only very high, pharmacologic

(supraphysiologic) doses of estrogen were tested. Effects of pharmacologic doses of estrogenic chemicals on prostate development are not predictive of effects at physiologic doses. The dramatic effects of physiologic doses of estrogens have been revealed in studies in which pregnant mice were administered low doses of E₂, the drugs diethylstilbestrol (DES) and ethinylestradiol, BPA, or the estrogenic insecticide methoxychlor, resulting in a permanent increase in prostate size in male offspring (Gupta 2000; Nagel et al. 1997; Thayer et al. 2001; vom Saal et al. 1997; Welshons et al. 1999). As the dose of E₂ or DES was increased into the pharmacologic range, the stimulating effect observed at low doses disappeared and inhibition of prostate development occurred (Gupta 2000; Timms et al. 2005; vom Saal et al. 1997). Thus, the inhibitory effects of pharmacologic

doses of estrogens on the developing prostate are opposite to effects of physiologic doses of the same estrogenic chemicals.

Available data on short-term effects of developmental estrogen exposures are consistent with the long-term effects observed in adulthood. For example, a high, pharmacologic dose of estradiol benzoate given to neonatal rats induced down-regulation of androgen receptor protein expression as early as postnatal day (PND) 6 (Prins and Birch 1995). In contrast, low, physiologically relevant doses of estrogenic chemicals (DES and BPA) fed to pregnant mice induced up-regulation of prostatic androgen receptor ligand binding activity in male offspring as early as PND3; this observation was replicated in organ culture of fetal mouse prostate treated with 0.1 pg/mL DES or 50 pg/mL BPA (Gupta 2000). The increase in prostate size in

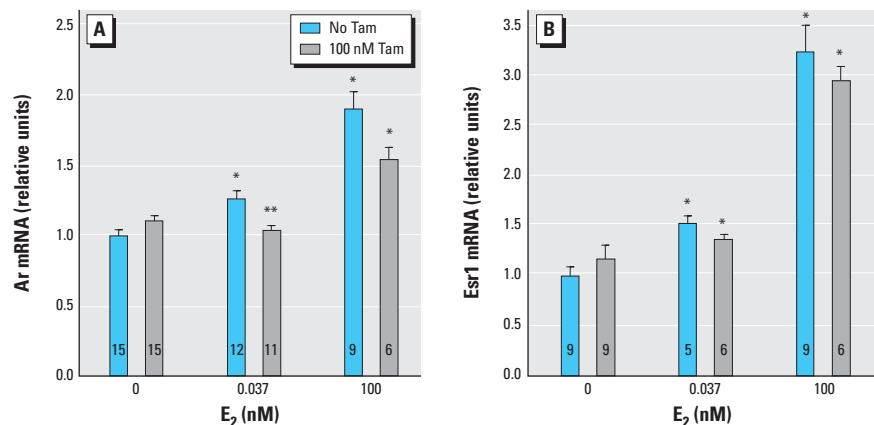


Figure 3. Antiestrogen treatment inhibits E₂-induced expression of *Ar* but does not significantly inhibit E₂-induced expression of *Esr1*. (A) The antiestrogen tamoxifen (Tam) blocks induction of *Ar* mRNA by a physiologic dose of E₂, and the inhibition by Tam is overcome by a pharmacologic dose of E₂. (B) Tam does not significantly inhibit induction of *Esr1* mRNA by a physiologic dose of E₂, or by a pharmacologic dose of E₂. Error bars represent 1 SE. The number of replicates measured for each treatment is shown in each bar. *Values significantly different from the control ($p < 0.05$). **Significant differences between the same E₂ treatment with and without Tam ($p < 0.05$).

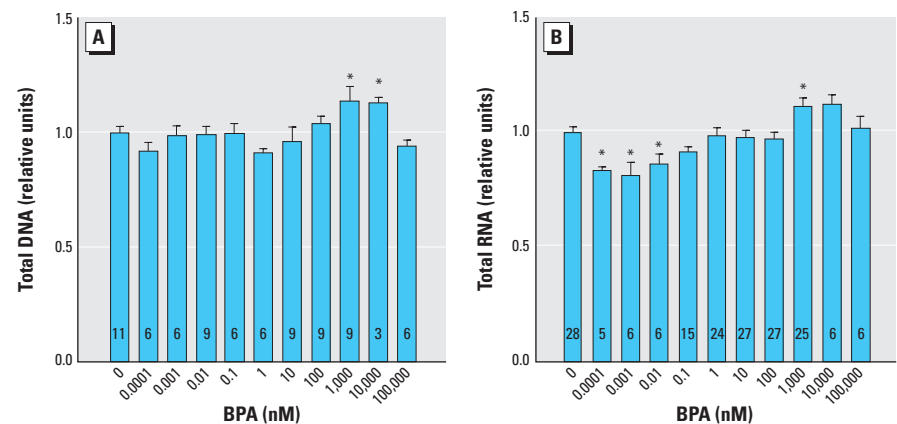


Figure 4. Indicators of cell growth in response to BPA. (A) Total DNA content was stable at low doses of BPA and significantly increased only at 1,000 nM. (B) Total RNA was significantly decreased at very low doses of BPA, 0.0001–0.001 nM, and significantly increased only at 1,000 nM. Error bars represent 1 SE. The number of replicates measured for each treatment is shown in each bar. *Values significantly different from the control ($p < 0.05$).

response to 50 pg/mL (0.22 nM) BPA in organ culture is below the threshold observed for stimulation of either *Ar* or *Esr1* gene expression observed in the present study.

Our findings support the hypothesis that prenatal exposure to elevated estrogen levels permanently increases prostate size and androgen responsiveness at least in part by inducing *Ar* mRNA expression. Importantly, the effects on *Ar* mRNA expression occurred with a threshold at 0.001 nM E_2 (0.28 pg/mL), consistent with concentrations previously shown to alter prostate development *in vivo*. Specifically, the total serum E_2 concentration in male mouse fetuses on GD18 is approximately 0.35 nM (94 pg/mL), and the free (unconjugated and unbound to plasma proteins) serum concentration of E_2 is 0.00077 nM (0.21 pg/mL), or 0.2% of total

serum E_2 (vom Saal et al. 1997), similar to findings in rats (Montano et al. 1995). An increase in free serum E_2 in male mouse fetuses (due to maternal administration of E_2 via Silastic capsule) to 0.0012 nM (0.31 pg/mL) significantly increased prostate size and the number of prostatic androgen receptors in adulthood (vom Saal et al. 1997). Our results show that at these same physiologic doses, E_2 acts directly on fetal UGS mesenchyme cells to increase *Ar* mRNA expression. This response was inhibited by the antiestrogens raloxifene (data not shown) and tamoxifen (Figure 3), suggesting that the induction of *Ar* mRNA by E_2 is mediated through the classical genomic estrogen receptor pathway.

The differences in the shapes of the dose-response curves for *Ar* and *Esr1* suggest that

the receptors are regulated by distinct mechanisms. Distinct dose-response relationships were also noted for vimentin, acidic ribosomal protein P0, total RNA content, and DNA content. These findings are consistent with data from microarray studies demonstrating considerable diversity in dose-response relationships of different estrogen-responsive genes; in particular, as one moves across the dose-response curve, entirely different sets of genes are activated or inhibited (Coser et al. 2003; Shioda et al. 2006). Induction of *Ar* and *Esr1* also displayed different responses to inhibition by antiestrogens, in that *Esr1* induction was not significantly inhibited by antiestrogen co-treatment (Figure 3). The threshold for effects on *Esr1* expression was between 0.01 nM (2.3 ng/mL) and 0.037 nM (8.4 ng/mL) E_2 (Figures 2B and 3B). This is above the normal range of free E_2 in serum in male mouse fetuses. However, serum estradiol may underestimate estrogen levels in prostate tissue because cells in the developing prostate express aromatase, which metabolizes testosterone to E_2 (Ellem and Risbridger 2006; Risbridger et al. 2003), and because estrogen receptor agonists, including xenoestrogens, exhibit additive effects in combination (Rajapakse et al. 2002). The induction of *Esr1* expression by E_2 suggests that estrogen exposure may create a positive feedback loop in the UGS, such that exposure to estrogens increases sensitivity to future or continuing exposure.

Although the observed effects of physiologic concentrations of E_2 on *Ar* mRNA expression are consistent with established *in vivo* effects, our pharmacologic dose range (e.g., 100 nM) *in vitro* observations are not consistent with established *in vivo* effects (Gupta 2000; Prins and Birch 1995; vom Saal et al. 1997), which predicted a decline in *Ar* mRNA expression relative to control levels at this high dose of E_2 (Figure 2A). The *in vivo* regulation of androgen receptors by pharmacologic doses of estrogens may thus involve systemic and posttranscriptional effects that are not observable in terms of *Ar* mRNA levels in isolated mesenchyme cells. The involvement of posttranscriptional mechanisms is supported by the observation that developmental exposure to pharmacologic doses of estrogens permanently up-regulates androgen receptor degradation by the proteasome (Woodham et al. 2003).

The behavior of BPA in this system is consistent with the established activity of BPA as an estrogen receptor agonist (reviewed by vom Saal and Hughes 2005; vom Saal and Welshons 2006; Welshons et al. 2006), which was first reported in 1936 (Dodds and Lawson). The weak effects of BPA on cell growth, measured as DNA and RNA content,

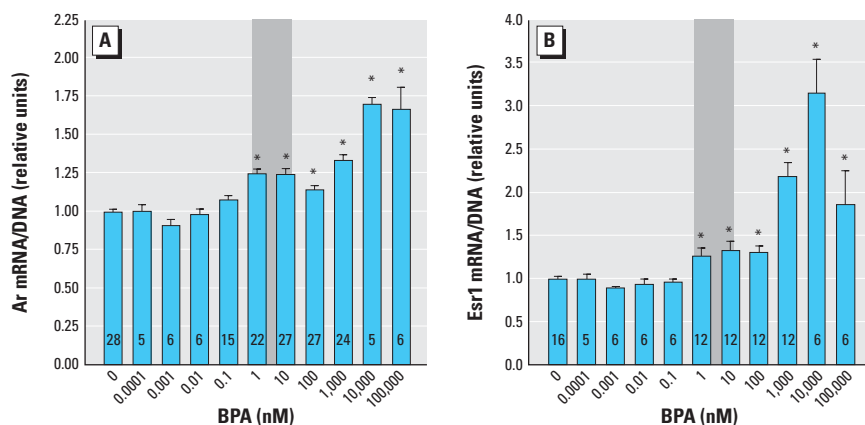


Figure 5. Gene expression of *Ar* and *Esr1* are induced by the synthetic estrogen BPA. (A) *Ar* mRNA expression increases with increasing dose of BPA. (B) Biphasic *Esr1* mRNA expression response increases with increasing dose of BPA up to a dose of 10,000 nM (10 μ M or 2.28 μ g/mL). Shaded areas represent the typical range of concentrations of unconjugated BPA measured in human serum (Welshons et al. 2006). Error bars represent 1 SE. The number of replicates measured for each treatment is shown in each bar.

*Values significantly different from the control ($p < 0.05$).

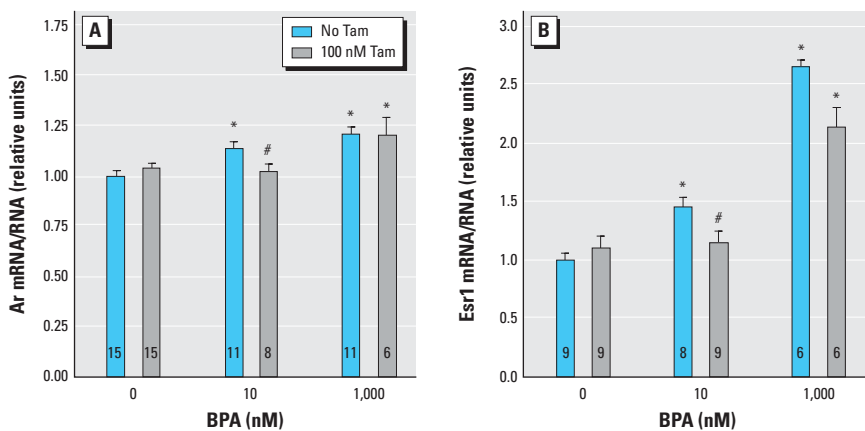


Figure 6. Antiestrogen treatment inhibits BPA-induced expression of steroid receptors *Ar* and *Esr1*. (A) The antiestrogen tamoxifen (Tam) blocks induction of *Ar* mRNA by a 10-fold lower dose of BPA, and the inhibition by tamoxifen is overcome by a 10-fold higher dose of BPA. (B) Tam inhibits induction of *Esr1* mRNA by a 10-fold lower dose of BPA, and the inhibition by Tam is overcome by a 10-fold higher dose of BPA. Error bars represent 1 SE. The number of replicates measured for each treatment is shown in each bar.

*Values significantly different from the control ($p < 0.05$). **Significant differences between the same BPA treatment with and without Tam ($p < 0.05$).

are consistent with previous reports that the relative potency of BPA is greater in stimulating estrogen receptor–dependent gene transcription than in stimulating growth of uterine tissue (Nagel et al. 2001). There are several interesting differences between the dose–response curves of *Ar* and *Esr1* in response to BPA compared with E₂. Based on the thresholds of induction of gene expression, BPA is approximately 1,000-fold less potent than E₂ for induction of *Ar*, but only about 30-fold less potent than E₂ for induction of *Esr1* (based on a threshold of *Esr1* induction of 0.037 nM E₂; Figure 3B). In addition, the shape of the dose–response curve for *Esr1* differs between E₂ and BPA; induction of *Esr1* by BPA was inhibited by tamoxifen, whereas induction of *Esr1* by E₂ was not significantly inhibited by tamoxifen. These differences between E₂ and BPA, which are seen in the *Esr1* dose–response curves but not the *Ar* dose–response curves, underline the probability that distinct mechanisms are at work in the induction of *Ar* and *Esr1* by estrogens.

Of great importance, the doses of BPA required for induction of both *Ar* and *Esr1* are within the range of typical levels of BPA measured in human serum, which range from approximately 1 to 10 nM (Figure 5) (Schönfelder et al. 2002; Welshons et al. 2006). Because our experiments measured the response to BPA in the absence of other estrogens, they are likely to underestimate the induction of *Ar* and *Esr1* expression in response to the additive mixture of endogenous estrogens, BPA, and other xenoestrogens to which humans are continuously exposed in our modern world (Colborn et al. 1993). The consequences of developmental induction of *Ar* and *Esr1* for the adult phenotype of the prostate have not been directly examined, but exposure during fetal life to very low doses of BPA (2–50 µg/kg/day) permanently increases prostate size in mice (Gupta 2000; Nagel et al. 1997). Neonatal exposure to a 10 µg/kg/day dose of BPA results in precancerous lesions (prostate interepithelial neoplasia) in adult male rats, associated with epigenetic changes (Ho et al. 2006). The report of Ho et al. (2006) is consistent with the finding that estrogenic chemicals stimulate an abnormal rate of proliferation in basal epithelial cells in the primary ducts of the mouse fetal prostate (Timms et al. 2005). Basal cells are progenitor cells proposed to be involved in prostate cancer (Kirschenbaum et al. 2006). We are currently examining whether the permanent increase in prostate AR receptor protein in mice exposed during fetal life to low doses of estrogenic chemicals is caused by a permanent increase in expression of the *Ar* gene, and whether this is associated with a change in DNA methylation at the *Ar* gene.

Conclusions

Ar mRNA in mesenchyme cells isolated from fetal mouse prostate is up-regulated by E₂ within its physiologic range, and by BPA within the range detected in human fetal serum. Induction of *Ar* mRNA by E₂ or BPA was inhibited by antiestrogen co-treatment. Therefore, the low-dose effects of estrogens on prostatic *Ar* regulation are estrogen receptor–dependent, act at the transcriptional level, are mediated through local effects on UGS mesenchyme cells, and can be modeled in a primary cell culture system. In contrast, down-regulation of androgen receptor protein in response to high doses of estrogens *in vivo* likely includes systemic and post-transcriptional mechanisms. *Esr1* mRNA is also up-regulated by E₂ and BPA in a dose-dependent manner, suggesting the possibility of positive feedback in estrogen effects on the prostate. The induction of *Esr1* by E₂ is not significantly inhibited by antiestrogen treatment, suggesting the involvement of non-estrogen receptor–mediated mechanisms.

Taken together, these results are consistent with the hypothesis that prenatal exposure to elevated estrogen or xenoestrogen levels within the physiologic range results in an increase in androgen receptor and estrogen receptor 1 (α) number in the developing prostate mesenchyme, which increases androgen and estrogen responsiveness and growth. The estrogen receptor–dependent induction of *Ar* by BPA confirms that this mechanism is not unique to E₂ and underscores the vulnerability of the developing reproductive system to the additive effects of exogenous estrogenic endocrine disruptors.

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Exposure to Organophosphates Reduces the Expression of Neurotrophic Factors in Neonatal Rat Brain Regions: Similarities and Differences in the Effects of Chlorpyrifos and Diazinon on the Fibroblast Growth Factor Superfamily

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BACKGROUND: The fibroblast growth factor (FGF) superfamily of neurotrophic factors plays critical roles in neural cell development, brain assembly, and recovery from neuronal injury.

OBJECTIVES: We administered two organophosphate pesticides, chlorpyrifos and diazinon, to neonatal rats on postnatal days 1–4, using doses below the threshold for systemic toxicity or growth impairment, and spanning the threshold for barely detectable cholinesterase inhibition: 1 mg/kg/day chlorpyrifos and 1 or 2 mg/kg/day diazinon.

METHODS: Using microarrays, we then examined the regional expression of mRNAs encoding the FGFs and their receptors (FGFRs) in the forebrain and brain stem.

RESULTS: Chlorpyrifos and diazinon both markedly suppressed *fgf20* expression in the forebrain and *fgf2* in the brain stem, while elevating brain stem *fgfr4* and evoking a small deficit in brain stem *fgf22*. However, they differed in that the effects on *fgf2* and *fgfr4* were significantly larger for diazinon, and the two agents also showed dissimilar, smaller effects on *fgf11*, *fgf14*, and *fgfr1*.

CONCLUSIONS: The fact that there are similarities but also notable disparities in the responses to chlorpyrifos and diazinon, and that robust effects were seen even at doses that do not inhibit cholinesterase, supports the idea that organophosphates differ in their propensity to elicit developmental neurotoxicity, unrelated to their anticholinesterase activity. Effects on neurotrophic factors provide a mechanistic link between organophosphate injury to developing neurons and the eventual, adverse neurodevelopmental outcomes.

KEY WORDS: brain development, chlorpyrifos, diazinon, fibroblast growth factor, fibroblast growth factor receptors, microarrays, neurotoxicity, organophosphate insecticides. *Environ Health Perspect* 115:909–916 (2007). doi:10.1289/ehp.9901 available via <http://dx.doi.org/> [Online 27 February 2007]

The developmental neurotoxicity of organophosphate pesticides represents a biological conundrum that has important ramifications for human exposures (for review see Colborn 2006; Costa 2006; Landrigan 2001; Milesion et al. 1998; Slotkin 2005; Weiss et al. 2004). All of the organophosphates produce systemic toxicity by inhibiting acetylcholinesterase, resulting in overt symptoms of cholinergic hyperstimulation; these effects have therefore been assumed to be the common mechanism underlying adverse developmental consequences (Milesion et al. 1998). However, the fetus and neonate recover from cholinesterase inhibition much more quickly than adults (Chakraborti et al. 1993; Lassiter et al. 1998), yet display greater overall toxicity and damage to the central nervous system (for review see Pope 1999; Slotkin 2004, 2005). Indeed, evidence accumulating over the past decade implicates a host of other mechanisms in the developmental neurotoxicity of the organophosphates that depend instead upon the direct targeting of events specific to the developing brain (for review see Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 2004). Importantly, many of these processes are vulnerable to organophosphates at doses below those necessary to elicit signs of

systemic toxicity and even below the threshold for significant inhibition of cholinesterase (Pope 1999; Slotkin 2004, 2005).

Although a wide variety of intermediate events in brain development connect the initial effects of organophosphates on neural cell differentiation to the eventual synaptic and behavioral defects (Pope 1999; Slotkin 2004, 2005), little information is currently available about specific cellular mechanisms that render the developing brain so vulnerable to these agents. Indeed, many events in differentiation and assembly of neural circuits are affected, including the processes of neuronal and glial cell replication and differentiation, specification of neurotransmitter phenotypes, axonogenesis and synaptogenesis, and synaptic function (Barone et al. 2000; Casida and Quistad 2004; Gupta 2004; Jameson et al. 2006; Pope 1999; Slotkin 1999, 2004). In turn, the diversity of these targets suggests that the organophosphates disrupt some very basic processes in neural cell differentiation. For that reason, a number of investigations have turned to the neurotrophic factors known to play critical roles in neural development and damage/repair processes.

In adults, fully symptomatic organophosphate poisoning produces peripheral

neuropathies and then a reactive increase in formation of neurotrophic factors mediating repair and neuritic outgrowth (Pope et al. 1995). Although we are dealing with events in the central nervous system rather than with peripheral neuropathies, it is not unreasonable to hypothesize that these factors are equally or even more important at the subtoxic exposures that damage the developing brain. Two sets of neurotrophic factors have been explored to date. First, acetylcholinesterase itself is thought to play a nonenzymatic role in neural development (Brimijoin and Koenigsberger 1999), and we recently demonstrated induction of the neurotoxic splice variant of acetylcholinesterase at organophosphate exposures below the threshold for detectable inhibition of enzymatic activity in neonatal rat brain after apparently subtoxic exposures to chlorpyrifos or diazinon (Jameson et al. 2007). In addition, two recent studies (Betancourt and Carr 2004; Betancourt et al. 2006) focused on nerve growth factor and brain-derived neurotrophic factor after exposure of newborn rats to chlorpyrifos or chlorpyrifos oxon, the active metabolite that inhibits cholinesterase. Although these researchers used exposures that were above the threshold for cholinesterase inhibition and somatic growth impairment, they found no significant decrease in either protein (Betancourt and Carr 2004) and only a small decrease (10–20%) in the mRNA encoding nerve growth factor (Betancourt et al. 2006).

Thus, if organophosphate effects on neurotrophic factors play an important role in the developmental neurotoxicity of these agents, then other factors are likely to be more highly affected. In the present study, we turned our attention to the large number of fibroblast growth factors (FGFs) and their

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This research was supported by grant ES10356 from the National Institutes of Health.

T.A.S. and F.J.S. have provided expert witness testimony on behalf of government agencies, corporations, and/or individuals. F.F. declares he has no competing financial interests.

Received 14 November 2006; accepted 27 February 2007.

receptors (FGFRs). The FGF superfamily plays a widespread and vital role in brain development and in the repair from neural injury (Dono 2003). Across the various stages of development, the FGFs promote and maintain neuronal cell replication and are required for differentiation into the terminal transmitter phenotype (Gage et al. 1995; Johe et al. 1996). The different FGFs play specific roles in neuronal cell differentiation, neurite outgrowth, and the recovery from damage in regions such as the striatum and hippocampus (Hart et al. 2000; Limke et al. 2003; Murase and McKay 2006; Ohmachi et al. 2000; Ray et al. 1993; Takagi et al. 2005). The same regions are known targets for the adverse neurodevelopmental effects of organophosphates (Barone et al. 2000; Slotkin 1999, 2004, 2005), which disrupt the very same cellular events for which the FGFs provide trophic signals (Axelrad et al. 2003; Das and Barone 1999; Howard et al. 2005; Song et al. 1998). Accordingly, we used a microarray approach to examine the family of FGFs and their receptors, comparing the effects of two different organophosphates, chlorpyrifos and diazinon, to emphasize points of similarity and difference: if the developmental neurotoxicity of the organophosphates involves neurotrophic mechanisms unrelated to the inhibition of cholinesterase, then there may be significant disparities in their impact on neurotrophic factors. We concentrated on doses that evoke barely detectable inhibition, too low to elicit any signs of cholinergic hyperstimulation (Slotkin et al. 2006b; Song et al. 1997); our assessments were conducted in two brain regions, the brain stem and forebrain, that differ both in anatomical attributes as well as in maturational timetables (Rodier 1988).

Materials and Methods

Animal treatments. All experiments were carried out in accordance with federal and state guidelines and with prior approval of the Duke University Institutional Animal Care and Use Committee; all animals were treated humanely and with due care for alleviation of distress. Timed-pregnant Sprague-Dawley rats (Charles River, Raleigh, NC, USA) were housed in breeding cages, with a 12-hr light/dark cycle and free access to food and water. On the day of birth, all pups were randomized and redistributed to the dams with a litter size of 9–10 to maintain a standard nutritional status.

Chlorpyrifos and diazinon (both from Chem Service, West Chester, PA, USA) were dissolved in dimethylsulfoxide to provide consistent absorption (Whitney et al. 1995), and were injected subcutaneously in a volume of 1 mL/kg body weight once daily on postnatal days (PNDs) 1–4; control animals received equivalent injections of dimethylsulfoxide

vehicle. For both agents, we used doses below the threshold for growth retardation and systemic toxicity (Campbell et al. 1997; Slotkin et al. 2006a; Whitney et al. 1995): 1 mg/kg for chlorpyrifos and either 1 or 2 mg/kg for diazinon. This chlorpyrifos treatment and the higher dose of diazinon produce neurotoxicity in developing rat brain while eliciting < 20% cholinesterase inhibition, whereas the lower dose of diazinon does not produce any detectable inhibition (Slotkin 1999, 2004; Slotkin et al. 2006b; Song et al. 1997; Whitney et al. 1995), or any of the symptoms of cholinergic hyperstimulation known to be characteristic of anticholinesterase activity (Clegg and van Gemert 1999). These treatments thus resemble the nonsymptomatic exposures reported in pregnant women (De Peyster et al. 1993) and are within the range of expected fetal and childhood exposures after routine home application or in agricultural communities (Gurunathan et al. 1998; Ostrea et al. 2002).

On PND5 (24 hr after the last dose), one male pup was selected from each of five litters in each treatment group. Animals were decapitated, the cerebellum was removed, and the brain stem and forebrain were separated by a cut made rostral to the thalamus. Tissues were weighed and flash-frozen in liquid nitrogen and maintained at -45°C until analyzed. Our study design involved the analysis of 40 separate tissues: one animal from each of five litters for each of the four treatment groups, with two tissues (brain stem, forebrain) from each animal.

Microarray determinations. Tissues were thawed and total RNA was isolated using the Aurum total RNA Fatty and Fibrous Tissue Kit (Bio-Rad Laboratories, Hercules, CA, USA). RNA quality was verified using the RNA 6000 LabChip Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). An aliquot of each sample used in the study was withdrawn and combined to make a reference RNA preparation to be included on each array. RNA amplification was carried out using a commercial kit (Low RNA Input Fluorescent Linear Amplification Kit; Agilent).

Each RNA sample was annealed with a primer containing a polydT and a T7 polymerase promoter. Reverse transcriptase produced a first and second strand cDNA. T7 RNA polymerase then created cRNA from the double stranded cDNA by incorporating cyanine-3- (for the reference RNA) or cyanine-5- (for the sample RNA) labeled cytidine 5-triphosphate; the quality of the labeled cRNA was again verified and the absolute concentration was measured spectrophotometrically. For each pair of reference cRNA and experimental cRNA hybridized to an array, equal amounts of cRNA (0.75 μg) were

hybridized using a commercial kit (*In situ* Hybridization Kit-Plus; Agilent). Hybridization was performed at 60°C for 17 hr with Agilent Whole Rat Genome Arrays (G4131A). The arrays were washed with Agilent's SSPE Wash Protocol using a solution of 6 \times SSPE, 0.005% N-lauroylsarcosine, a solution of 0.06 \times SSPE, 0.005% N-lauroylsarcosine, and Agilent's Stabilization and Drying Solution. The arrays were scanned on an Agilent G2565BA Microarray Scanner, and data from the scans were compiled with Agilent Feature Extraction Software 8.1. The steps from RNA amplification through extraction of the scanner output data were performed by a private contractor (Cogenics, Research Triangle Park, NC, USA).

Array normalizations and error detection were carried out using Silicon Genetics' GeneSpring GX Version 7.2 (Agilent), via the Enhanced Agilent Feature Extraction Import Preprocessor. First, values of poor quality intensity and low dependability were removed using a "filter on flags" feature, where standardized software algorithms determined which spots were "present," "marginal," or "absent"; spots were considered "present" only where the output was uniform, not saturated and significant above background, whereas spots that satisfied the main requirements but were outliers relative to the typical values for the other genes were considered "marginal." Filters were set to retain only the values that were found to be present or marginal for further analysis; however, of the genes that passed the filter, none was marginal.

Data were normalized in three steps using the algorithms supplied with the Feature Extraction software. The first step divides the signal in the Cy5 channel (sample RNA) by that in the Cy3 channel (reference RNA), to give the measured ratio for each gene in the array. The second normalization adjusts the total signal of each chip to a standard value ("normalize to 50th percentile") determined by the median of all the reliable values on the chip; this renders the output of each chip comparable with that of every other chip in the study. The third normalization step is applied to each gene across all the arrays in the study ("normalize to median"); The median of all the values obtained for a given gene is calculated and used as the normalization standard for that gene, so that, regardless of absolute differences in the expression of the various genes, they are placed on the same scale for comparison.

After normalization, one final quality-control filter was applied in which genes showing excessive biologic variability were discarded; the criterion for retention was that more than half of the eight treatment \times region groupings had to have coefficients of variation < 30%.

For some of the genes, the arrays contained multiple probes and/or replicates of the

same probe in different locations on the chip, and these were used to verify the reliability of values and the validity of the measures on the chip. In these cases, to avoid artificially inflating the number of positive findings, we limited each gene to a single set of values, selecting those obtained for the probe showing the smallest intragroup (treatment, region) variance; the other values for that gene were used only to corroborate direction and magnitude of change. Through these procedures we identified five defective arrays with sequential production numbers, for which one corner of the array showed a nonuniform overall difference in brightness that affected the readings in that region of the chip. The affected samples were reevaluated on replacement arrays that did not repeat the problem. Our experimental design ensured that the replacement readings were distributed among all the treatment groups because our sample sequence was control, chlorpyrifos, diazinon 1 mg/kg, diazinon 2 mg/kg; thus we did not run the risk of generating a spurious apparent treatment effect from differences among arrays. The defective arrays did allow us to perform an additional quality-control evaluation because most of the spots on the defective arrays were in the portion that did not show the defect. Comparing the values on the replacement arrays to the valid portions of the defective arrays produced a close correspondence of values (correlation coefficient = 0.98).

Statistical procedures. Because of the requirement to normalize the data across arrays and within each gene, the absolute values for a given gene are meaningless; only the relative differences between regions and treatments can be compared. Accordingly, results for the regional differences in gene expression in control rats are presented as means \pm SEs of the normalized ratios for each gene, but the effects of the treatments are given as the percentage change from control to allow for visual comparison of the relative changes evoked for each gene, regardless of its control ratio. However, statistical comparisons were based on the actual ratios (log-transformed because the data are in the form of ratios) rather than the percent change.

Our design involved planned comparisons of the organophosphate-exposed groups to the controls and between the two different organophosphates, so it was important to consider the false positive rate and to protect against type 1 errors from repeated testing of the same database. Accordingly, before looking at effects on individual genes, we performed a global analysis of variance (ANOVA) incorporating all treatments, both regions, and all genes in a single comparison. Lower-order ANOVAs were then carried out as permitted by the interactions of treatment with region and gene that justified subdivisions of

the data set. Finally, differences for individual treatments for a specified gene in a single brain region were evaluated with Fisher's protected least significant difference test. However, where there was no treatment \times region interaction for a given gene; only the main treatment effect was reported without subtesting of effects in individual regions. For ANOVA results, effects were considered significant at $p < 0.05$ (two-tailed, because we were interested in both increases and decreases in gene expression). In addition to these parametric tests of the direction and magnitude of changes in gene expression, we evaluated the incidence of significant differences as compared with the false positive rate using Fisher's exact test, applying a one-tailed criterion of $p < 0.05$ because only an increase above the false positive rate would be predicted. Finding a significant decrease in the incidence of detected differences relative to the false positive rate would be biologically implausible and statistically meaningless.

Results

Of the FGF and FGFR genes present on the microarray, 19 genes passed the quality control filters, encoding 15 of the FGFs and all 4 FGFRs (Table 1). In control rats, we did not observe any overall pattern of regional preference for expression of these genes: Of the 19 genes evaluated, only 6 showed significant regional differences, with *fgf9*, *fgf22*, and *fgfr2* more highly expressed in the brain stem, whereas *fgf14*, *fgf20*, and *fgfr1* were higher in the forebrain. Organophosphate exposures elicited significant, regionally selective changes in gene expression for the FGFs and FGFRs. Multivariate ANOVA (all treatments, all genes, both regions) showed a significant treatment \times region \times gene interaction ($p < 0.0001$), enabling separate evaluations for each gene.

Out of the 19 genes, 7 displayed significant main treatment effects or an interaction of treatment \times region, as compared with an expected false positive rate of only 1 gene ($p < 0.02$).

For the genes encoding FGFs, chlorpyrifos exposure produced a significant overall decrement (main treatment effect, $p < 0.05$) and specific reductions in the expression of *fgf2*, *fgf11*, *fgf20*, and *fgf22* (Figure 1). By far, the largest effect was on *fgf20*, which showed a 50% deficit in the forebrain; this region also displayed a significant deficit in *fgf2* and *fgf11*. In contrast, the brain stem showed smaller decreases restricted to *fgf2* and *fgf22*.

The effects of diazinon on the FGF genes displayed similarities to those of chlorpyrifos, but also some differences. The lower dose of diazinon caused a large reduction in forebrain *fgf20* expression as did chlorpyrifos, but diazinon failed to decrease forebrain *fgf2* or *fgf11* significantly, and instead evoked a reduction in *fgf14* (Figure 2A). In the brain stem, we again saw a small decrease in *fgf2* and *fgf22*. Increasing the dose of diazinon to 2 mg/kg produced a further divergence from the effects seen with chlorpyrifos (Figure 2B). Although we still saw a significant reduction in *fgf20* in the forebrain, no other gene was significantly affected for this region. In the brain stem, the higher dose of diazinon produced an even larger decrease in *fgf2* expression than with either chlorpyrifos or the lower diazinon treatment. These regional differences between diazinon and chlorpyrifos were statistically significant ($p < 0.02$ for the interaction of treatment \times region \times gene).

Two of the four genes encoding the FGFRs, *fgfr1* and *fgfr4*, showed statistically significant treatment-related changes in expression, but the magnitude of the effect on *fgfr1* was quite small, $< 10\%$ (Figure 3). In contrast,

Table 1. Control values.

Name	Gene	Genbank accession no.	Brainstem	Forebrain
Fibroblast growth factor 1	<i>fgf1</i>	NM_012846	0.98 \pm 0.05	1.00 \pm 0.04
Fibroblast growth factor 2	<i>fgf2</i>	NM_019305	1.17 \pm 0.09	1.02 \pm 0.03
Fibroblast growth factor 3	<i>fgf3</i>	NM_130817	1.13 \pm 0.11	0.87 \pm 0.04
Fibroblast growth factor 9	<i>fgf9</i>	NM_012952	1.37 \pm 0.08	0.81 \pm 0.02*
Fibroblast growth factor 11	<i>fgf11</i>	NM_130816	1.03 \pm 0.13	1.18 \pm 0.10
Fibroblast growth factor 12	<i>fgf12</i>	NM_130814	1.02 \pm 0.07	1.01 \pm 0.03
Fibroblast growth factor 13	<i>fgf13</i>	NM_053428	0.97 \pm 0.02	1.00 \pm 0.02
Fibroblast growth factor 14	<i>fgf14</i>	NM_022223	0.87 \pm 0.06	1.20 \pm 0.04*
Fibroblast growth factor 15	<i>fgf15</i>	NM_130753	1.04 \pm 0.15	1.15 \pm 0.20
Fibroblast growth factor 17	<i>fgf17</i>	NM_019198	0.96 \pm 0.06	1.00 \pm 0.08
Fibroblast growth factor 18	<i>fgf18</i>	NM_019199	0.96 \pm 0.04	0.99 \pm 0.04
Fibroblast growth factor 20	<i>fgf20</i>	NM_023961	0.92 \pm 0.23	1.63 \pm 0.22*
Fibroblast growth factor 21	<i>fgf21</i>	NM_130752	0.99 \pm 0.08	0.97 \pm 0.07
Fibroblast growth factor 22	<i>fgf22</i>	NM_130751	1.11 \pm 0.02	0.93 \pm 0.04*
Fibroblast growth factor 23	<i>fgf23</i>	NM_130754	1.10 \pm 0.10	0.84 \pm 0.10
Fibroblast growth factor receptor 1	<i>fgfr1</i>	NM_024146	1.00 \pm 0.02	1.08 \pm 0.02*
Fibroblast growth factor receptor 2	<i>fgfr2</i>	BF 557.572	1.10 \pm 0.04	0.96 \pm 0.02*
Fibroblast growth factor receptor 3	<i>fgfr3</i>	NM_053429	0.98 \pm 0.02	1.00 \pm 0.03
Fibroblast growth factor receptor 4	<i>fgfr4</i>	XM_344570	0.89 \pm 0.03	0.98 \pm 0.03

GenBank accession numbers from GenBank (2007).

*Significant difference between brain stem and forebrain.

fgfr4 showed significant increases in expression for all three organophosphate treatment groups, an effect that was restricted to the brain stem. Diazinon produced a larger increase than did chlorpyrifos. Again, the regional differences in the effects of the two organophosphates were statistically distinguishable ($p < 0.05$ for the interaction of treatment \times region \times gene).

Earlier work with higher doses of chlorpyrifos administered for longer periods of time—

treatments that evoke significant and persistent cholinesterase inhibition and/or growth impairment—identified small (10–20%) decreases in the mRNA encoding nerve growth factor (Betancourt et al. 2006). We also examined expression of the two corresponding genes on our arrays, *ngfb* (GenBank accession no. XM_227525; GenBank 2007) and *ngfg* (GenBank NM_031523) but found only a small (6%) decrease in *ngfb* in the forebrain

that did not achieve statistical significance (data not shown). Similarly, we found no significant effects on expression of the gene encoding brain-derived neurotrophic factor (*bdnf*; Genbank accession no. NM_012513; data not shown).

Discussion

Our results show that neonatal exposure to doses of organophosphates that are below the threshold for any signs of systemic intoxication or growth deficits, and just at the threshold for any detectable inhibition of cholinesterase, nevertheless causes profound suppression of several members of the FGF superfamily of neurotrophic factors. Indeed, the effects for chlorpyrifos or diazinon in the present study are far larger than those reported previously for other neurotrophic factors, even when the earlier work involved chlorpyrifos treatments at higher doses for longer periods, producing much greater cholinesterase inhibition or frank growth impairment (Betancourt and Carr 2004; Jameson et al. 2007). Furthermore, we found a distinct regional hierarchy corresponding to the maturational and anatomical differences between the brain stem and the forebrain (Rodier 1988). The brain stem matures earlier than the forebrain and contains a high proportion of cell bodies for cholinergic, catecholaminergic, and serotonergic neurons; the forebrain develops later and contains the terminal projections of these neurotransmitter systems, all of which are prominent targets for the developmental neurotoxicity of organophosphates (Slotkin 1999, 2004, 2005). In keeping with this regional specificity, *fgf20* was suppressed by chlorpyrifos or diazinon in the forebrain, whereas the two organophosphates differentially targeted *fgf2* in the brain stem (diazinon > chlorpyrifos). There were also smaller effects on *fgf11*, *fgf14*, *fgf22*, *fgfr1*, and *fgfr4*, each of which also displayed either a regionally selective effect or a difference between the two organophosphates. In contrast, no such regional differences were reported for other neurotrophic factors such as nerve growth factor or acetylcholinesterase splice variants associated with neural damage/repair (Betancourt and Carr 2004; Jameson et al. 2007). Indeed, to obtain any effect on nerve growth factor gene expression, the dose and duration of chlorpyrifos exposure have to be increased to the point where cholinesterase is persistently inhibited and/or growth is impaired; even then, there is only a small (10–20%) decrement (Betancourt et al. 2006). In the present study, we used lower doses and shorter durations of exposure that caused barely detectable cholinesterase inhibition and no growth impairment, and found no significant deficits for either nerve growth factor or brain-derived neurotrophic factor, indicating that selective members of the FGF superfamily

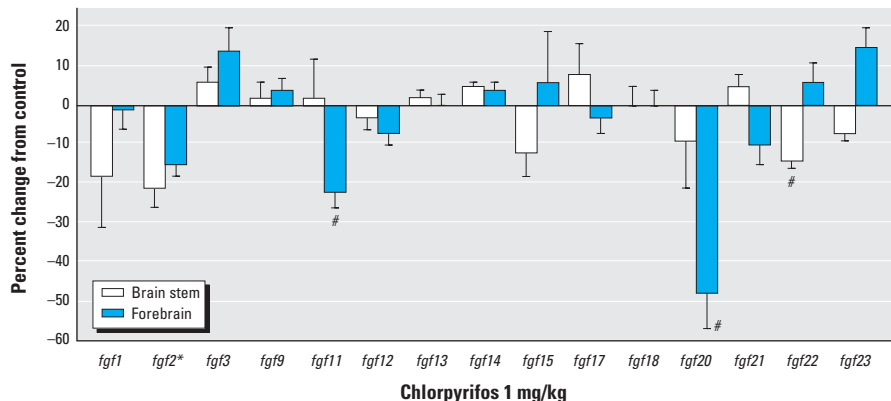


Figure 1. Effects of 1 mg/kg/day chlorpyrifos exposure (PNDs1–4) on expression of genes encoding the FGFs, shown as the percentage change from control values (Table 1). Multivariate ANOVA (all genes, both regions) indicates a main effect of treatment ($p < 0.05$) and interactions of treatment \times gene ($p < 0.03$) and treatment \times region \times gene ($p < 0.02$). Error bars indicate SE. *Significant main treatment effect. #Significant difference from corresponding control region after a treatment \times region difference was detected by ANOVA.

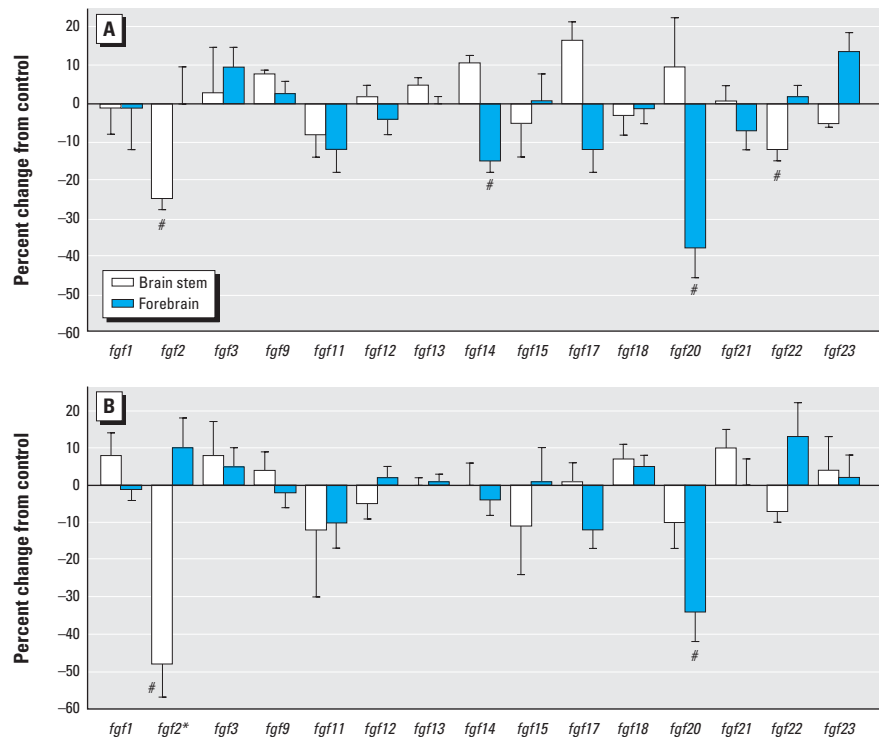


Figure 2. Effects of diazinon exposure on PNDs 1–4 at 1 mg/kg/day (A) or 2 mg/kg/day (B) on expression of genes encoding the FGFs, shown as the percentage change from control values (Table 1). Multivariate ANOVA (all genes, both regions) indicates a significant interaction of treatment \times region \times gene ($p < 0.003$). Error bars indicate SE. *Significant main treatment effect. #Significant difference from corresponding control region after a treatment \times region difference was detected by ANOVA.

are indeed far more sensitive to disruption by the organophosphates.

The regional selectivity suggests that the effects of neonatal exposure to organophosphates on expression of FGFs reflects the targeting of specific processes in brain development rather than a global interference with neurotrophic responses. Below, we will consider each of the FGFs in turn, emphasizing their various roles in neural development and plasticity. However, first it is necessary to consider the important differences between chlorpyrifos and diazinon.

In the developing rat brain, treatment with 1 mg/kg chlorpyrifos produces approximately a 10–20% inhibition of cholinesterase (Song et al. 1997), roughly equivalent to that seen at 2 mg/kg diazinon (Slotkin et al. 2006b); the lower dose of diazinon (1 mg/kg) produces no significant inhibition whatsoever (Slotkin et al. 2006b). If the effects of these agents were the result of cholinesterase inhibition, then the chlorpyrifos treatment should produce the same pattern of effects as the higher dose of diazinon, whereas the lower diazinon dose should have no effect at all. In fact, though, all three treatments shared the same major suppression of *fgf20* in the forebrain. Furthermore, the low dose of diazinon inhibited brain stem *fgf2* and *fgf22* expression, and increased *fgfr4*, just as did chlorpyrifos. It is therefore apparent that these effects are totally unrelated to cholinesterase inhibition, the mechanism that underlies the systemic toxicity of the organophosphates, thus reinforcing the concept that the developmental neurotoxicity of these agents represents a separable set of mechanisms that operate at lower exposures (Colborn 2006; Slotkin 1999, 2004, 2005; U.S. Environmental Protection Agency 2006).

It is also noteworthy that we saw several important differences in the effects of chlorpyrifos as compared with diazinon: chlorpyrifos decreased *fgf2* and *fgf11* in the forebrain, whereas diazinon did not; in the brain stem, diazinon reduced *fgf2* and induced *fgfr4* much more than did chlorpyrifos, and also had effects on forebrain *fgf14* and *fgfr1* that were not seen with chlorpyrifos. The similarities and disparities suggest that the two organophosphates are likely to produce many parallels in subsequent neurodevelopmental deficits but may also differ in important ways. Although much more information is available for chlorpyrifos than for diazinon, several findings already suggest differential targeting of neural cell replication, neuritic outgrowth, cytotoxic events, and cholinergic and monoaminergic neurotransmitter systems by these two agents (Jameson et al. 2007; Qiao et al. 2001; Slotkin et al. 2006a, 2007).

The two specific members of the FGF superfamily that were most highly affected by

neonatal organophosphate exposure were *fgf2* and *fgf20*, both of which have clearly established roles in neurodevelopment, plasticity, damage/repair, and neurodegenerative disorders. The expression of *fgf2* shows spatial and temporal relationships to the maturational profile of each brain region (Gomez-Pinilla et al. 1994; Monfils et al. 2006) and up-regulation of this gene is required for the recovery from developmental brain injury (Monfils et al. 2005; Yoshimura et al. 2001). In addition, we recently found that *fgf2* is intimately involved in the programming of neural plasticity associated with less injurious perturbations, such as prenatal stress (Fumagalli et al. 2005). Accordingly, the robust down-regulation of this gene caused by neonatal organophosphate treatment is likely to play an important role in the neurodevelopmental outcomes of such exposures; in particular, our finding of regional selectivity (brain stem > forebrain) is in keeping with the targeting of a specific maturational stage and/or anatomical location and similarly, the preferential sensitivity to diazinon predicts a potentially worsened outcome with this agent. For *fgf2*, our finding of gross suppression by organophosphates during the brain growth spurt (Dobbing and Sands 1979) is highly likely to have long-term, adverse consequences for neural development and behavioral function. Even a brief period of *fgf2* down-regulation interferes directly with neurogenesis (Tao et al. 1997), and the period of exposure studied here (first few days after birth) corresponds to the peak proliferation period in a number of neuronal populations, including those of the hippocampal dentate gyrus (Kempermann et al. 1997; Rodier 1988). If similar effects occur with organophosphate exposures in earlier or later developmental periods, this could explain why shifting the exposure window often targets the neural cells and

regions that are undergoing the most rapid development (Slotkin 1999, 2004, 2005).

In contrast to the organophosphate-evoked reduction in *fgf2*, which was more prominent in the brain stem, the suppression of *fgf20* was selective for the forebrain. What is particularly notable about the regional difference is that *fgf20* is preferentially expressed in a subregion of the forebrain, the striatum (Ohmachi et al. 2000), which contains the majority of dopamine projections, the loss of which results in Parkinson disease. There is growing suspicion that repeated developmental exposures to pesticides that target striatal dopamine projections play a significant role in the later emergence of this neurodegenerative disorder (for review, see Cory-Slechta et al. 2005; Landrigan et al. 2005). Indeed, the relationship of suppressed *fgf20* expression to dopaminergic deficits and thence to Parkinson disease is directly supported by human genetic data (Murase and McKay 2006; Takagi et al. 2005; van der Walt et al. 2004) and by the specific role of this neurotrophic factor in promoting survival of the very neurons that are lost in Parkinson disease (Damier et al. 1999; Yamada et al. 1990).

The requirement for *fgf20* is similarly found for development of these neurons and for preventing their death from apoptosis secondary to oxidative stress (Murase and McKay 2006); the striatum is especially sensitive to oxidative damage, in part because dopamine itself produces oxidative metabolites (Hirsch 1994; Olanow and Arendash 1994). Organophosphates target striatal dopamine systems by causing release of dopamine while simultaneously evoking oxidative stress through other cellular mechanisms (Bloomquist et al. 2002; Gupta 2004; Jett and Navoa 2000; Karen et al. 2001; Lazarini et al. 2004; Slotkin et al. 2002, 2005; Slotkin and Seidler 2007). Consequently,

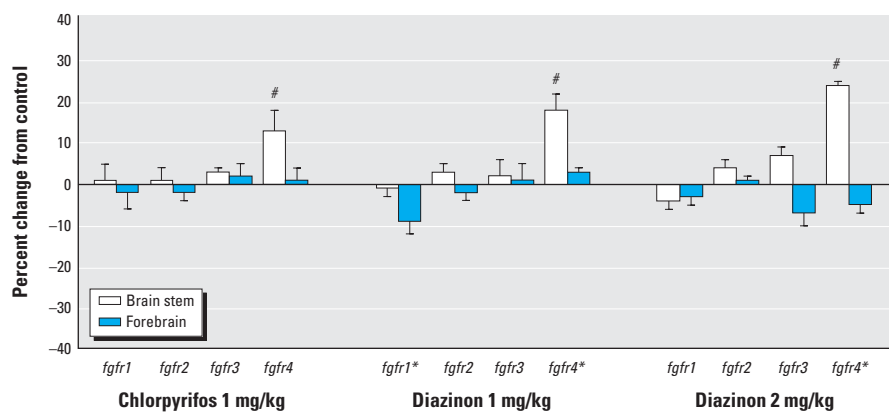


Figure 3. Effects of chlorpyrifos or diazinon exposure on expression of genes encoding the FGF receptors, shown as the percentage change from control values (Table 1). Multivariate ANOVA (all treatments, all genes, both regions) indicates interactions of treatment \times region ($p < 0.005$) and treatment \times region \times gene ($p < 0.05$). Error bars indicate SE.

*Significant main treatment effect. #Significant difference from corresponding control region after a treatment \times region difference was detected by ANOVA.

these neural pathways are among the most vulnerable to long-term damage after developmental exposure to chlorpyrifos (Aldridge et al. 2005b; Dam et al. 1999; Slotkin et al. 2002, 2007; Slotkin and Seidler 2007). Finally, it should be noted that *fgf2* is also deficient in dopaminergic neurons in Parkinson disease (Tooyama et al. 1993) and *fgf2* and *fgf20* actually act in concert to protect dopaminergic neurons from oxidative injury and to promote their repair (Kriegstein et al. 1998; Ohmachi et al. 2000; Otto and Unsicker 1990; Shults et al. 2000), so that the combined deficit in both these neurotrophic factors, superimposed on organophosphate-induced oxidative stress, may render striatal dopamine pathways especially vulnerable. In particular, in Parkinson disease, degeneration begins in the brain stem (Braak et al. 2006), the region in which we found reduced *fgf2* expression after neonatal organophosphate exposure. We therefore anticipate that later in life, exposed individuals may show a greater likelihood of neurodegenerative disorders such as Parkinson disease, which is already known to be associated with pesticide exposures in adulthood (Kamel and Hoppin 2004).

In addition to the major changes seen for *fgf2* and *fgf20* expression, we found significant but smaller effects on other members of the FGF superfamily, including *fgf11*, *fgf14*, and *fgf22*, and also on two of the receptor genes, *fgfr1* and *fgfr4*. Although the roles for these are less well understood, there is substantial evidence for involvement of all of them in neurodevelopment and hence in the developmental neurotoxicity of the organophosphates. Developing neurons show particularly high expression of *fgf11* and *fgf14* (Luo et al. 2002; Wang et al. 2000), and the latter participates directly in neuronal signaling, axonal trafficking, and development of sodium channels required for neuronal excitability (Lou et al. 2005; Wang et al. 2002). Deficits in *fgf14* are associated with the development of movement disorders (Wang et al. 2002) and it is well established that early exposure to organophosphates compromises the subsequent development of motor activity (Carr et al. 2001; Dam et al. 2000). Similarly, *fgf22*, a recently discovered member of the FGF family, is involved in neural differentiation of granule cells and acts as an organizer of presynaptic activity (Umemori et al. 2004). Again, hippocampal and cerebellar granule cells are known to be targeted by developmental exposure to organophosphates (Abdel-Rahman et al. 2003; Roy et al. 2005) in association with profound alterations in the patterns of presynaptic neuronal activity and associated behaviors (Aldridge et al. 2005b; Dam et al. 1999; Icenogle et al. 2004; Levin et al. 2001, 2002; Qiao et al. 2003, 2004; Richardson

and Chambers 2004, 2005; Slotkin et al. 2001, 2002; Slotkin and Seidler 2007).

Of the two FGF receptor genes for which we found significant changes, *fgfr1* is only weakly expressed in the developing brain but probably serves as an organization factor (Blak et al. 2005); we found only a small effect on this receptor, restricted to diazinon, so that this may ultimately contribute to some differences in outcome between the two organophosphates. We saw a far more robust effect on *fgfr4*, again with a greater action of diazinon as compared to chlorpyrifos. In contrast to *fgfr1*, *fgfr4* is highly expressed in developing brain, especially in the hippocampus (Cool et al. 2002; Limke et al. 2003; Wright et al. 2004) and in cholinergic neurons of the medial habenular nucleus (Miyake and Itoh 1996), and is involved specifically in neurite outgrowth (Hart et al. 2000). Furthermore, this receptor binds *fgf2*, one of the FGF members highly affected by organophosphate exposure, reinforcing the greater potential contribution of this particular signaling pathway; indeed, the fact that *fgfr4* was up-regulated suggests that this is a partial, adaptive response to the suppression of *fgf2* expression, a conclusion reinforced by the fact that diazinon was more effective than chlorpyrifos for both the down-regulation of brain stem *fgf2* and the up-regulation of *fgfr4*. In keeping with these relationships, developmental exposure to organophosphates especially targets each of the processes associated with *fgfr4*: neurons of the cholinergic phenotype (Slotkin 1999, 2004, 2005), the hippocampus (Abdel-Rahman et al. 2003; Pung et al. 2006; Roy et al. 2005; Terry et al. 2003), and neuritic outgrowth (Axelrad et al. 2003; Das and Barone 1999; Howard et al. 2005; Slotkin et al. 2006a; Song et al. 1998).

It is important to note a number of limitations of our approach, which relies on measurement of gene expression at the mRNA level assessed in two, broadly defined brain regions. First, the magnitude of changes was small when compared to the fold-change that can be obtained with *in vitro* studies of pesticide neurotoxicity, where typically one assesses the effects on a single cell type at a fixed stage of differentiation (Mense et al. 2006). That is hardly surprising, given the heterogeneity of the brain stem and the forebrain, so that even a large change in gene expression in a specific cell population would be "washed out" by mRNA from unaffected areas. In fact, treatment of animals with higher doses of organophosphates that produce outright toxicity or even lethality rarely produces changes in gene expression exceeding 10–30% *in vivo* (Betancourt et al. 2006; Damodaran et al. 2006a, 2006b).

The second limitation is inherent in any study of mRNA: this measure by itself does

not provide a definitive answer about the actual rate of synthesis and degradation of the encoded protein, the factors that actually control the concentrations of the corresponding trophic factors or factors within the cell. Nevertheless, there are two important aspects designed into the current study that render the findings relevant and interpretable. First, we included chlorpyrifos as a test compound whose impact has already been confirmed for the relevant end points of neural cell differentiation, axonogenesis, and other developmental processes known to be regulated by FGFs. Second, our interpretations rely on patterns of changes across multiple regions and FGF/FGFR subtypes rather than on a single change in one region. Nevertheless, it is obvious that a direct mechanistic link needs to be established between the changes seen here at the mRNA level and the known outcomes for chlorpyrifos, or the suspected outcomes for diazinon.

Our results are also limited by the fact that we examined only males, whereas there are numerous studies showing significant sex differences in the outcomes of developmental exposure to organophosphates (Aldridge et al. 2004, 2005a, 2005b; Dam et al. 2000; Levin et al. 2001; Ricceri et al. 2006; Slotkin 2005; Slotkin et al. 2001, 2002; Slotkin and Seidler 2007). Here, we were limited primarily by practical considerations of technical capabilities and cost. We expect, however, that males and females will show important differences in transcriptional profiles in accordance with the sex-selective nature of organophosphate-induced neurodevelopmental anomalies. Despite these limitations, though, our results are likely to be relevant to environmental exposures of fetuses and children to organophosphates. Although our studies were modeled primarily on the upper limits of estimated or measured exposures after home or agricultural application (Gurunathan et al. 1998; Ostrea et al. 2002), recent studies indicate that much lower, long-term exposures of pregnant women result in adverse neurodevelopmental outcomes for their children (Eskenazi et al. 2007; Rauh et al. 2006; Young et al. 2005). Accordingly, modeling the potential mechanisms underlying the adverse effects of these agents at exposures below the threshold for cholinesterase inhibition may provide important insights into the etiology of these orders and thus to potential strategies for amelioration.

In conclusion, our results show that neonatal exposure to low doses of organophosphates, below the threshold for any signs of systemic toxicity and spanning the threshold for any detectable cholinesterase inhibition, evoke profound and regionally selective effects on expression of specific members of the FGF superfamily of neurotrophic factors, with the largest effects seen for *fgf2* and *fgf20*.

The fact that there are similarities but also notable disparities in the responses to chlorpyrifos and diazinon, and that robust effects were seen at a dose of diazinon that does not inhibit cholinesterase, supports the idea that organophosphates differ in their propensity to elicit developmental neurotoxicity, unrelated to their anticholinesterase activity. Further, the specific involvement of *fgf2* and *fgf20* in development of the hippocampus and striatum matches some of the most sensitive regional targets for neurodevelopmental disruption by the organophosphates, reinforcing the potential mechanistic role of suppression of these neurotrophic factors in organophosphate-induced developmental neurotoxicity.

The close relationship between deficiencies in these factors and loss of dopamine neurons in Parkinson disease further indicates the need for long-term studies of the effects of early organophosphate exposure, preferably occupying the entire life span so as to determine whether developmental exposures lead to later emergence of neurodegenerative disorders. Finally, the identification of specific neurotrophic factors targeted by organophosphates may enable the design of targeted, interventional strategies that might prevent or offset neurodevelopmental damage in cases of known exposure. For example, increasing the concentration of FGF2 protein appears to offset the functional outcome of neonatal damage to the motor cortex (Monfils et al. 2005), and the neuroprotective effect of nicotine in animal models of Parkinson disease (Quik and Di Monte 2001) is associated with its ability to up-regulate *fgf2* expression (Belluardo et al. 2004). The characterization of neurotrophic factors involved in the developmental neurotoxicity of organophosphates thus establishes a mechanistic link between the initial events in neural cell damage and the eventual outcome, while at the same time providing valuable information to enable discrimination between the effects of different organophosphates as well as potential therapeutic interventions to prevent or offset neural damage.

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Reduction in Urinary Arsenic Levels in Response to Arsenic Mitigation Efforts in Araihaazar, Bangladesh

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BACKGROUND: There is a need to identify and evaluate an effective mitigation program for arsenic exposure from drinking water in Bangladesh.

OBJECTIVE: We evaluated the effectiveness of a multifaceted mitigation program to reduce As exposure among 11,746 individuals in a prospective cohort study initiated in 2000 in Araihaazar, Bangladesh, by interviewing participants and measuring changes in urinary As levels.

METHODS: The interventions included *a*) person-to-person reporting of well test results and health education; *b*) well labeling and village-level health education; and *c*) installations of 50 deep, low-As community wells in villages with the highest As exposure.

RESULTS: Two years after these interventions, 58% of the 6,512 participants with unsafe wells (As \geq 50 μ g) at baseline had responded by switching to other wells. Well labeling and village-level health education was positively related to switching to safe wells (As < 50 μ g/L) among participants with unsafe wells [rate ratio (RR) = 1.84; 95% confidence interval (CI), 1.60–2.11] and inversely related to any well switching among those with safe wells (RR = 0.80; 95% CI, 0.66–0.98). The urinary As level in participants who switched to a well identified as safe (< 50 μ g As/L) dropped from an average of 375 μ g As/g creatinine to 200 μ g As/g creatinine, a 46% reduction toward the average urinary As content of 136 μ g As/g creatinine for participants that used safe wells throughout. Urinary As reduction was positively related to educational attainment, body mass index, never-smoking, absence of skin lesions, and time since switching (*p* for trend < 0.05).

CONCLUSIONS: Our study shows that testing of wells and informing households of the consequences of As exposure, combined with installation of deep community wells where most needed, can effectively address the continuing public health emergency from arsenic in drinking water in Bangladesh.

KEY WORDS: arsenic, Bangladesh, epidemiology, environmental epidemiology, intervention. *Environ Health Perspect* 115:917–923 (2007). doi:10.1289/ehp.9833 available via <http://dx.doi.org/> [Online 5 February 2007]

Arsenic is abundant in the earth's crust and can be released to groundwater under certain conditions. In many parts of the world where groundwater is an important source of drinking water, As exposure has been linked to increased risks of skin lesions, skin cancer, internal cancers, and cardiovascular diseases (Chen et al. 1988, 1996; Haque et al. 2003). Widespread As exposure from drinking water in Bangladesh and several neighboring countries, in particular, is presently a public health emergency (Chakraborti et al. 2003). It has been estimated that > 50 million people have been chronically exposed to As by drinking groundwater with As concentrations exceeding the World Health Organization (WHO) standard (10 μ g/L) in Bangladesh alone (British Geological Survey 1999). Given the potential health consequences of As exposure, there is a need to identify and evaluate an effective mitigation policy that could potentially be implemented at the national scale.

Arsenic mitigation in Bangladesh is a multifaceted public health problem, requiring consideration of geological, engineering,

economical, and cultural constraints. There still is considerable debate about the technical advantages and pitfalls of various mitigation options in Bangladesh. Remediation options such as piped groundwater, rainwater harvesting, pond-sand filters, and the use of dug wells (Anstiss et al. 2001; Berg et al. 2006; Hassan 2005; Hoque et al. 2000, 2004) have been tested, but the record to date shows that they may not be safe, affordable, or sufficiently convenient (Ahmed et al. 2006). These remediation options, all of which require considerable maintenance, also deviate from the currently much more widespread practice of relying on hand-pumped tube wells, shallow or deep. This is a concern because, from a public health standpoint, emergency interventions are best accomplished through an existing technology that has already been accepted (Smith et al. 2000), even if an adjustment to existing behavior is required. Several studies have indicated that intervention programs using health education and/or well labeling increase the awareness of As-related health problems (Hadi 2003; Hanchett et al. 2002).

However, the effectiveness of such programs in reducing As exposure has rarely been evaluated at a large scale.

In 2000, researchers from Columbia University (CU) and partner institutions in Bangladesh established a large epidemiologic cohort study of 11,746 men and women to prospectively evaluate long-term health effects of As exposure through in-person biennial follow-up visits. At the same time, a mitigation program was initiated to promote switching to safe wells in order to reduce the continuing As exposure in the population. The impact of some of the component interventions evaluated on the basis of interviews has been reported previously for subsamples of the cohort (Madajewicz et al. 2006; Opar et al. 2007; Schoenfeld 2006). In this article we document for the first time the effectiveness of the mitigation program in terms of As exposure directly by comparing As concentration in the urine of cohort members at baseline and 2 years later. We also assess various host factors that may further influence urinary arsenic reduction in a subpopulation.

Methods

The Health Effects of Arsenic Longitudinal Study. The principal aim of the Health Effects of Arsenic Longitudinal Study (HEALS) is to investigate health effects of As exposure from drinking water in a well-defined geographic area of 25 km² in Araihaazar, Bangladesh. Details of the study methodologies have been presented elsewhere (Ahsan et al. 2006a;

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We thank our staff, field workers, and study participants in Bangladesh, without whom this work would not have been possible.

This research was supported by grants P42ES10349, P30ES09089, and ES000260 from the National Institute of Environmental Health Sciences, and grants R01CA107431, R01CA102484, and CA016087 from the National Institutes of Health.

The authors declare they have no competing financial interests.

Received 16 October 2006; accepted 5 February 2007.

Parvez et al. 2006; van Geen et al. 2002). Briefly, before subject recruitment into the cohort, water samples were collected in two stages and geographic coordinates were recorded for each well using handheld global positioning system (GPS) receivers. During the first stage (Figure 1), 4,999 contiguous wells in the area were sampled in March–June 2000 (van Geen et al. 2002, 2003b). During the second stage, 978 wells were sampled in a contiguous region during November–December 2001 (van Geen et al. 2003b). Demographic information about the users of the 5,966 wells was collected during both stages to provide a sampling framework for HEALS (Parvez et al. 2006).

Eligibility criteria for recruitment in the cohort study during the baseline visits included being married (in order to increase stability of residence), being at least 18 years of age, and having resided in the study area for ≥ 5 years. Upon verification of identity and eligibility of subjects, the interviewers explained the details of the study objectives and procedures. Because the average educational attainment was low in the population (50% did not have formal education), verbal consent was obtained from each eligible respondent who agreed to participate in the study. Participants were given the option of participating with or without donating a blood/urine sample (Ahsan et al. 2006a). A total of 11,746 men and women (a participation rate of 97.5%) were recruited at baseline into the HEALS cohort between October 2000 and May 2002. The HEALS cohort has since been followed at 2-year intervals.

The first 2-year follow-up visit took place between June 2002 and June 2004 (Figure 1). At both baseline and follow-up visits, detailed information on demographics, lifestyles, and well-water drinking history was collected with in-person interviews. Trained physicians who were blind to participants' As exposure status completed a comprehensive physical examination (Ahsan et al. 2006a). A spot urine sample was collected in 50-mL acid-washed tubes at baseline and the follow-up visits for 95.6% and 94.5% of the cohort participants, respectively. The study protocol and field procedures were approved by the CU Institutional Review

Board and by the Ethical Committee of the Bangladesh Medical Research Council.

Arsenic mitigation under HEALS. Several As mitigation programs were implemented by the HEALS investigators, in part concurrently with the baseline recruitment of participants and follow-up (Figure 1).

In-person communication of test results and health education at baseline. After the completion of baseline physical examinations and interviews, all study participants received an individual health education session from trained interviewers concerning As concentrations in their well and potential health impacts related to As exposure. Participants who consumed well water with As concentrations ≥ 50 $\mu\text{g/L}$ were specifically advised to switch, if possible, to nearby safer well(s), defined as wells with As concentrations < 50 $\mu\text{g/L}$. Participants who consumed well water with As concentrations < 50 $\mu\text{g/L}$ were not advised to switch wells.

Well labeling and education campaign at the village level. During January–June 2001, in the area where the first stage of well water sampling took place, metal placards with As concentrations were posted on each well after testing. During the same period, an education campaign was launched at the village level. A team of three educators traveled from village to village. Through the use of skits, songs, and focus-group discussions, they disseminated information on health problems related to As exposure in drinking water, the ineffectiveness of various popular ways of As removal (e.g., boiling water), the importance of switching wells to reduce As exposure, and the meaning of the metal placards posted on the wells. The team also advised that people with unsafe wells should change to a well with a lower level of As if a safe well (based on the Bangladesh standard of As < 50 $\mu\text{g/L}$) was not available in the vicinity (Madajewicz et al. 2006). There was no village-level education in the smaller area where the second stage of well-water sampling took place, and these wells were not labeled until 2004 when the follow-up survey was completed.

Installations of deep low-As community wells. From 2001 to 2004, but primarily in 2003, a total of 50 deep, low-As community

wells were installed across the 25-km² study area, generally in villages where As exposure was particularly high. A description of household response to the first 7 of these community wells has been described elsewhere (van Geen et al. 2003a). The depths of these 50 low-As wells ranged from 36 to 180 m; all community wells met the Bangladesh standard for As of 50 $\mu\text{g/L}$, and only two did not also meet the WHO guideline for As of 10 $\mu\text{g/L}$ (Opar et al. 2007).

Independently of CU and its local partners, most wells within the study area were painted red or green in 2003 after testing with Hach field kits by NGO (nongovernmental organization) workers hired under the Bangladesh Arsenic Mitigation and Water Supply Program (BAMWSP; 2007). Relative to the national standard for arsenic in drinking water of 50 $\mu\text{g/L}$, these results agreed with our laboratory tests for 88% of a randomly selected subset of 799 wells (van Geen et al. 2005). The inconsistencies were primarily underestimates in the 50–100 $\mu\text{g/L}$ range of arsenic concentrations that resulted in unsafe wells being labeled as safe.

Measurements of As exposure. Water samples from all 5,966 tube wells in the study area were collected in 60-mL acid-washed bottles after pumping each well for 5 min (van Geen et al. 2003b). Total As concentrations were first determined by graphite furnace atomic-absorption spectrometry (GFAA) with a Hitachi Z-8200 system (Hitachi, Tokyo, Japan) at the Lamont-Doherty Earth observatory of CU (van Geen et al. 2002). Water samples found to have As concentrations at or below the detection limit of GFAA (5 $\mu\text{g/L}$) were later reanalyzed by inductively-coupled plasma-mass spectrometry, which has a detection limit of 0.1 $\mu\text{g/L}$ (Cheng et al. 2004).

All urine samples collected at baseline and at follow-up visits were analyzed for total As concentration by GFAA using the Analyst 600 graphite furnace system (PerkinElmer, Wellesley, MA, USA), as previously described (Nixon et al. 1991). Urinary creatinine was analyzed using a method based on the Jaffe reaction for adjustment of urinary total As concentration (Yu et al. 2002). The concentration of total As in urine has often been used as an indicator of recent exposure because urine is the main route of excretion of most arsenic species. Therefore, we consider urinary As concentration to be a good measure of changes in As exposure over time.

Statistical analysis. We evaluated the determinants of switching wells in participants with safe and unsafe wells separately at baseline, because only the participants with unsafe wells were advised to switch. We defined a “safe well” according to the Bangladesh standard of < 50 $\mu\text{g/L}$ As in drinking water. Because switching wells is a dichotomized outcome and

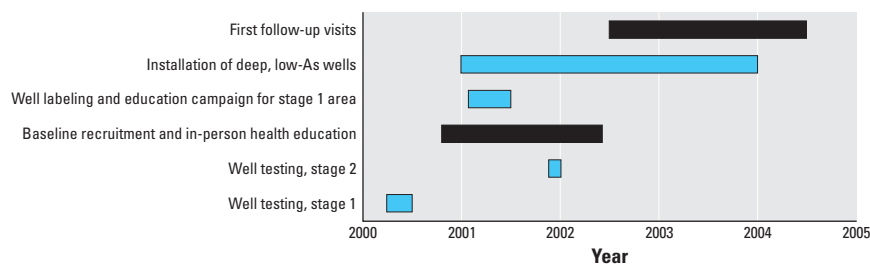


Figure 1. Timeline of HEALS activities. Bars indicate time period for activities; black bars indicate collection of urine samples.

the analysis involved follow-up time, we used Cox proportional hazard models to compare the likelihood of switching wells among groups with different attributes. We computed rate ratios (RR) for any well switching in participants with safe wells at baseline, and RRs for switching to known safe wells in participants with unsafe wells at baseline. A total of 423 participants had either died ($n = 104$) or moved ($n = 270$) since recruitment or were lost at the time of the follow-up survey ($n = 49$); therefore, their well-switching status was treated as censored. We calculated person-years of observation from the date of baseline visit to the date of well switching (reported at the follow-up) for those who switched wells, to the date of follow-up visit for those who did not switch, to death date and date of move reported by close relatives or neighbors for those who had died and moved, respectively. For the 49 subjects who were lost to follow-up, person-years of observation were considered from baseline to the midpoint between baseline and follow-up. Sensitivity analysis was conducted by excluding these subjects, and results did not change appreciably (data not shown). We included a total of 11,280 participants in this analysis (96% of the overall participants); those with unknown values on any of the covariates ($n = 466$) were excluded from the analysis.

Urinary arsenic is a continuous variable, and therefore multiple linear regression models were conducted to assess changes in urinary As level by baseline well-As level and switching status at follow-up. The underlying assumption of the models was that the relationship between variables is linear. Models were also run with log-transformed urinary As values; the results were similar and therefore are not shown. Participants with data on urinary As at both visits, known well-switching status, age, body mass index (BMI), and sex ($n = 10,645$; 90% of the overall participants) were considered in this analysis. Those who were excluded from the analysis did not differ appreciably from those included in the analysis with respect to demographic and lifestyle factors and arsenic exposure attributes (data not shown). To evaluate host factors that may influence urinary As reductions, we included participants with unsafe wells at baseline who switched to known safe wells ($n = 1,517$) because well As concentration at baseline and follow-up could be statistically held constant for this group. All analyses were performed using SAS, version 8.0 (SAS Institute Inc., Cary, NC, USA).

Results

As previously described (Ahsan et al. 2006b), the study population in general had a low educational level and included more females and middle-aged participants (Table 1). The distribution of sex, age, educational attainment, or

land ownership does not differ appreciably by baseline well-As categories. Well labeling and the health education campaign at baseline covered a greater proportion of participants with low As exposure. The proportion of participants with unsafe and safe wells that switched to a new source of drinking water averaged 58% and 17%, respectively (Table 1). Among participants with unsafe wells, most participants that changed their source of water switched to safe wells that were either labeled or installed by CU, or installed by an NGO/DPHE (Department of Public Health Engineering) (27%). The next largest proportion of participants with unsafe wells switched to tube wells that had not been sampled at baseline (23%). Among participants with safe wells at baseline, the majority of participants

who changed their source of water switched to a new tube well or another safe well labeled by CU. The majority (83%) of participants with unsafe wells at baseline who later switched wells did so because their wells were unsafe, whereas the majority (64%) of participants with safe wells at baseline who later switched wells stated they did so for convenience.

Among participants with unsafe wells at baseline, those with 5–9 and ≥ 10 years of education were more likely to switch to safe wells (wells labeled by CU, installed by CU, or installed by an NGO/DPHE), compared with those with < 5 years of education; the associated RRs were 1.36 [95% confidence interval (CI), 1.17–1.58] and 1.61 (95% CI, 1.36–1.74), respectively (Table 2). Land ownership, on the other hand, was inversely related

Table 1. Baseline and follow-up characteristics by baseline well As levels.

Characteristic ^a	< 50 µg/L ($n = 5,234$)	≥ 50 µg/L ($n = 6,512$)	<i>p</i> -Value
Baseline characteristics			
Percent male	42.8	43.0	0.78
Years of age (%)			
17–39	60.6	60.4	
40–59	37.2	37.3	
60–75	2.2	2.3	
Mean \pm SD	37.1 \pm 10.1	37.0 \pm 10.1	0.58
Health education/well labeling (%)			< 0.01
Yes	85.4	78.8	
Acres of land owned (%)			0.08
0	49.4	51.8	
≤ 1	32.2	30.6	
> 1	16.0	15.6	
Amount unknown	2.3	2.0	
Years of education (%)			0.58
0	44.3	44.9	
< 6	29.3	29.8	
6–9	15.2	14.7	
≥ 10	11.2	10.7	
Mean \pm SD	3.5 \pm 3.9	3.4 \pm 3.8	0.09
Mean well As (µg/L)	14.9	171.1	< 0.01
Presence of As-related skin lesions (%)	4.4	9.4	< 0.01
Creatinine-adjusted urinary As [µg/g creatinine (mean \pm SD)]	141.7 \pm 115.7	397.1 \pm 324.0	< 0.01
Distance to the nearest safe well [meters (mean \pm SD)]	14.8 \pm 13.7	48.5 \pm 38.7	< 0.01
Follow-up characteristics			
Time since baseline [months (mean \pm SD)]	23.5 \pm 5.3	25.3 \pm 7.0	0.39
Creatinine-adjusted urinary As [µg/g creatinine (mean \pm SD)]	136.3 \pm 108.1	291.9 \pm 265.6	< 0.01
Changes in creatinine-adjusted urinary As [µg/g creatinine (mean \pm SD)] ^b	-6.2 \pm 107.4	-108.6 \pm 319.9	< 0.01
Switching status (%)			
Did not switch	82.7	41.9	< 0.01
Switched to a safe well labeled by CU	5.7	20.3	
Switched to a community well installed by CU	0.0	4.1	
Switched to an NGO well	0.4	2.4	
Switched to an unsafe well labeled by CU	0.9	8.7	
Switched to new tube wells	10.4	22.6	
Reasons for switching, within those who switched (%)			
Well not safe	5.8	83.4	< 0.01
Smell/taste not good	5.7	1.5	
Deleterious relations with neighbor/owner	5.7	0.8	
Well no longer exists	4.2	1.3	
Well does not work	14.7	3.2	
For convenience	63.8	9.7	

^aData on education level were missing for 5 subjects with safe wells (< 50 µg/L As) and for 4 subjects with unsafe wells (≥ 50 µg/L As) at baseline; data were unknown on baseline skin lesion status for 138 and 75 subjects, respectively; data were missing for baseline urinary As for 378 and 148 subjects; data were missing on distance to the nearest safe well for 136 and 101 subjects; data were missing on follow-up urinary As for 340 and 299 subjects; and data were missing on well-switching status for 314 and 271 subjects, respectively. ^bChanges in creatinine-adjusted urinary As = follow-up – baseline.

Table 2. Associations of switching wells with sociodemographics and As-related variables.

Baseline characteristic	Participants with a safe well at baseline ^a			Participants with an unsafe well at baseline ^a		
	Switched to any well		RR for switching (95% CI)	Switched to a safe well		RR for switching to a safe well (95% CI)
	Yes (% of total) ^b	Total ^b		Yes (% of total) ^b	Total ^b	
Education (years)						
0	16.3	2,241	1.00	23.4	2,777	1.00
1–4	18.6	1,485	1.17 (0.98–1.37)	25.1	1,867	1.14 (1.01–1.28)
5–9	15.1	768	0.96 (0.77–1.19)	28.2	916	1.36 (1.17–1.58)
≥ 10	13.7	554	0.92 (0.71–1.19)	30.1	672	1.61 (1.36–1.92)
Land owned (acres)						
0	17.8	2,505	1.00	25.3	3,216	1.00
≤ 1	16.2	1,616	0.92 (0.78–1.07)	25.9	1,920	0.93 (0.83–1.04)
> 1	14.0	813	0.83 (0.67–1.04)	24.8	977	0.83 (0.71–1.01)
Amount unknown	9.7	114	0.53 (0.29–0.97)	21.9	119	0.75 (0.51–1.11)
Baseline skin lesion status						
No	16.2	4,828	1.00	25.0	5,656	1.00
Yes	22.7	220	1.56 (1.16–2.10)	28.5	576	1.15 (0.99–1.38)
Health education and well labeling						
No	17.1	756	1.00	16.8	1,338	1.00
Yes	16.4	4,292	0.80 (0.66–0.98)	27.7	4,894	1.84 (1.60–2.11)
Baseline well As (μg/L)						
< 10	16.1	2,631	1.00			
10–24	17.5	1,006	1.07 (0.90–1.28)			
25–49	16.6	1,411	0.97 (0.82–1.14)			
50–99				23.0	1,988	1.00
100–299				25.6	3,433	1.38 (1.23–1.55)
300–499				29.3	724	1.62 (1.37–1.90)
≥ 500				34.5	87	1.85 (1.26–2.72)
Distance to nearest safe well (m)						
≥ 100	0.0	10	1.00	11.3	648	1.00
50–99	13.2	211		20.8	1,691	2.22 (1.72–2.86)
25–49	18.5	552	1.38 (0.90–2.09)	30.0	1,886	3.71 (2.90–4.73)
< 25	16.4	4,275	1.22 (0.83–1.78)	29.2	2,007	3.95 (3.09–5.06)

^aRRs were adjusted for all variables in the table and additionally for age and sex. A total of 11,280 subjects were included in the analysis; participants with unknown information for any of the covariates were excluded from the analysis. ^b“Total” indicates the number of participants with the attribute, and “% of total” indicates the percentage of persons with that attribute that switched wells.

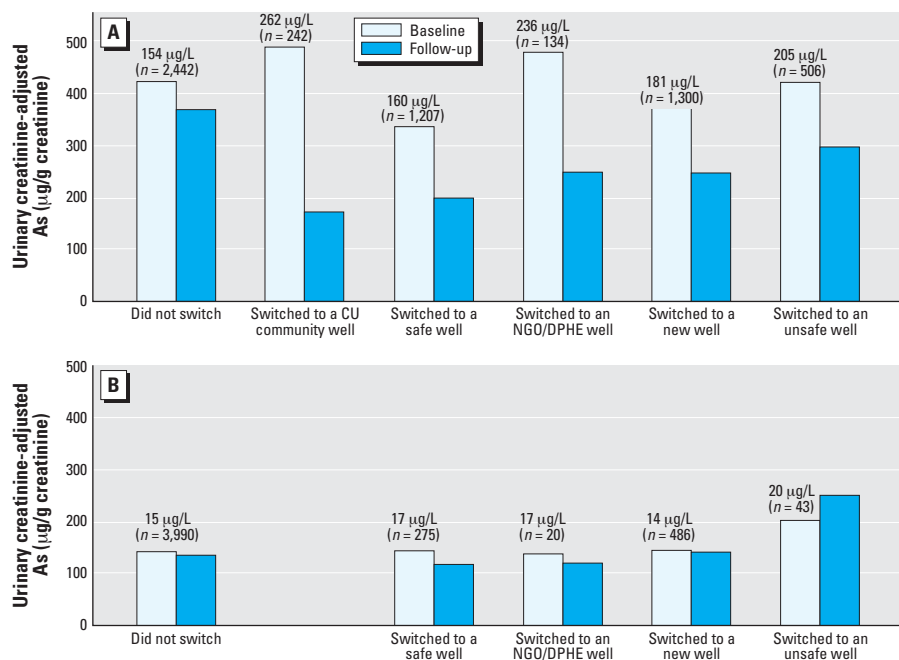


Figure 2. Mean urinary creatinine-adjusted As levels for participants with an unsafe well at baseline (A) and for those with a safe well at baseline (B). Additional adjustments were made for age, sex, and BMI. Values above the bars are average baseline well As concentration and number.

to switching to safe wells, although not at the statistically significant level of $p < 0.05$. Well labeling and the village-level health campaign were positively associated with subsequent switching to safe wells (RR = 1.84; 95% CI, 1.60–2.11). Higher baseline As concentration was also positively related to the likelihood of switching to safe wells. An estimate of the distance from each unsafe well to the nearest safe well was calculated on the basis of the available GPS data (van Geen et al. 2002). Participants with unsafe wells located within 50 m of a safe well were approximately 4 times more likely to switch to safe wells compared with participants with an unsafe well located ≥ 100 m from a safe well. Among participants with safe wells at baseline, well labeling and the village-level health campaign were inversely related to switching wells (RR = 0.80; 95% CI, 0.66–0.98) (Table 2). In this group, no apparent relationships were observed between switching wells and educational attainment, baseline well As level, or distance to the nearest safe well among participants with safe wells. In both groups, the presence of lesions led to a somewhat higher proportion of switching wells (Table 2).

At baseline, urinary As concentration was on average nearly 3 times higher for participants using unsafe wells (397 $\mu\text{g As/g creatinine}$) compared with those using safe wells (141 $\mu\text{g As/g creatinine}$; Table 1). Average concentrations of As in unsafe and safe well water differed by more than an order of magnitude (171 and 15 $\mu\text{g/L}$, respectively). At follow-up, urinary As levels in participants with unsafe wells at baseline dropped by 109 $\mu\text{g As/g creatinine}$ (Table 1). This reduction is attributable to switching wells (Figure 2). The average drop of urinary As in participants who switched to a safe well ranged from 29 to 65%, according to the types of wells participants switched to, with an overall average drop of 46% from 375 to 200 $\mu\text{g As/g creatinine}$. Most significantly, the urinary As level dropped from 491 to 172 $\mu\text{g As/g creatinine}$ in participants who indicated that they had switched to deep, low-As community wells (Figure 2). The urinary As level in participants who switched to a new well or another unsafe well also decreased. Urinary As levels did not change appreciably in the population that continued to rely on a safe well or in participants with an unsafe well at baseline who had not switched to a different well (Figure 2).

The determinants of changes in urinary As were examined in greater detail for the 1,517 participants with unsafe wells who switched to known safe wells. We controlled for baseline urinary As level, baseline well As level, and well As level at the time of follow-up in the analysis to evaluate the influences of host factors on changes in urinary As in excess of what can be explained by differences in As exposure. The

reduction in urinary creatinine-adjusted As was significantly greater in men (p for trend = 0.01) and in participants who had never smoked (p for trend = 0.03), had a higher BMI (p for trend = 0.01), had higher education (p for trend = 0.01), and had no skin lesions at baseline ($p = 0.04$) (Table 3). The drop in urinary As increased with time elapsed since switching (p for trend = 0.02) but reached a plateau after a duration of ≥ 12 months since switching wells. Within participants who switched from an unsafe to a safe well, the reduction in urinary As did not differ significantly by the distance to the nearest safe well, land ownership, or the status of well labeling and village-level health education.

Discussion

The high proportion of the Bangladesh population that is exposed to arsenic by drinking water from tube wells remains a public health emergency. The present study is the first large prospective analysis to examine the effectiveness of an As intervention program in terms of well-switching behavior and changes in urinary As concentrations.

Among participants with unsafe wells at baseline, 58% switched to other wells at follow-up. The extent of well switching we recorded in the study area for the entire 2002–2004 period is consistent with smaller but more rapid surveys conducted in the same study area in 2002 (Madajewicz et al. 2006) and in 2004 (Opar et al. 2007). In a portion of Araihsar adjacent to the study area, where wells were tested under BAMWSP but the message was not reinforced through additional health education, only 27% of households stopped using 1,870 wells that had been tested to be unsafe (Schoenfeld 2006). In other parts of Bangladesh where blanket testing was conducted by UNICEF, 38% of the test population switched from 6,359 unsafe wells (Sarker et al. 2005). These comparisons suggest that our team's continued presence in the study area significantly encourages switching of wells. The significant positive association between well labeling/village-level health campaigns and switching to safe wells among participants with unsafe wells (Table 2) confirms the reinforcing effect of these additional efforts.

Participants with unsafe wells who switched to new wells that were not tested by CU, but were possibly tested by BAMWSP, on average did not increase their exposure to As (Figure 2). However, the urinary As levels of these participants indicate that a significant number of these wells probably contain > 50 $\mu\text{g/L}$ As. These data emphasize that well testing should be made available on demand at the village level. The drop in urinary As among participants with unsafe wells who switched to a different unsafe well suggests that they sought

wells with a lower As content than their own, albeit still unsafe. This is an argument for not only labeling wells as safe or unsafe but also for indicating each well's actual As level, as was done in our study area. The dose–response relationship between baseline well As level and switching behavior among participants with unsafe wells at baseline (Table 2) also suggests that participants take into account the actual As concentration that was measured and not only its safe/unsafe status.

Consistent with previous analyses of sub-population in the study area (Madajewicz et al. 2006; Opar et al. 2007; Schoenfeld 2006), we found that switching wells drops off rapidly when safe wells are located ≥ 100 m away. The largest drop in urinary As observed in those villages where participants benefited from the

installation of a community well is consistent with the consumption of water with high As (mean 258 $\mu\text{g/L}$) at baseline and < 10 $\mu\text{g/L}$ at follow-up (van Geen et al. 2006). In view of the particularly beneficial effect of community wells that are periodically monitored (van Geen et al. 2006), the spatial density of such wells in different villages should be calculated to minimize the number of households that live > 100 m from a safe water source. The large existing database of close to 5 million well tests compiled under BAMWSP could be used effectively to produce such estimates and help target those aquifers that are systematically low in As (van Geen et al. 2006).

We observed positive relationships of switching to safe wells and urinary As reduction with educational attainment but not with

Table 3. Determinants of urinary As changes (follow-up – baseline) among participants with an unsafe baseline well who switched to a safe well ($n = 1,517$).

Characteristics	No.	Adjusted changes in urinary creatinine-adjusted As ^a		<i>p</i> -Values for trend tests
		Mean	SD	
Sex				
Female	885	–171.4	13.0	0.01
Male	632	–186.4	12.4	
Education (years)				
0	621	–159.2	13.6	0.02
1–4	451	–170.1	13.7	
5–9	249	–165.0	15.2	
≥ 10	196	–200.6	16.6	
Land owned (acres)				
0	790	–162.0	11.0	0.93
≤ 1	470	–160.3	11.8	
> 1	234	–155.6	13.9	
Amount unknown	23	–216.9	33.0	
Age (years)				
< 30	385	–166.2	15.0	0.45
30–39	511	–175.9	14.1	
40–49	403	–165.5	13.8	
≥ 50	218	–187.2	15.7	
BMI				
< 17.6	402	–155.5	14.2	0.02
17.6–19.2	401	–172.9	14.6	
19.3–21.5	349	–186.3	14.4	
≥ 21.6	365	–180.1	14.3	
Baseline smoking status				
Never-smokers	999	–189.6	13.0	0.03
Past smokers	93	–171.2	19.3	
Current smokers	425	–160.3	14.7	
Baseline skin lesion status				
No	1,357	–186.8	11.8	0.04
Yes	160	–160.6	16.4	
Health education and well labeling				
No	217	–172.7	15.4	0.73
Yes	1,300	–174.7	12.1	
Time since switching wells (months)				
< 6	126	–160.3	17.0	0.03
6–11	117	–156.2	18.4	
12–17	364	–180.1	14.2	
18–23	507	–185.1	13.9	
≥ 24	403	–186.8	15.0	
Distance to the nearest safe well (m)				
< 25	560	–166.1	13.0	0.44
25–49	548	–180.9	12.9	
50–99	339	–171.8	14.4	
≥ 100	70	–176.0	21.7	

^aFollow-up – baseline; means were adjusted for all variables in the table and baseline urinary creatinine-adjusted As, baseline well As, and well As level in the wells participants switched to.

land ownership (Tables 2 and 3). Persons with higher educational attainment may be more responsive to health education and intervention. On the other hand, persons who own land may be less likely to switch wells because they may tend to use their own well located in the property. Such relationships between well switching and different indicators of socioeconomic status need to be considered in the plan and design of intervention programs.

BMI was positively related to urinary As reduction. A high BMI in Bangladesh is an indicator of a better nutrition status, which may influence the excretion of As. Smoking of tobacco products and presence of skin lesions were inversely associated with the reduction of total urinary As, indicating that these factors may be related to a higher body burden of As or a reduced clearance of As from the body. These observations are consistent with our previous findings of a synergistic effect of high level of As exposure with tobacco smoking and low BMI on the risk of skin lesions (Ahsan et al. 2006b; Chen et al. 2006).

It is worth noting that there was considerable overlap between the timing of the various interventions and when the baseline data were collected (Figure 1). Wells were labeled and participants were exposed to village-level health education before collection of the majority of baseline urine samples. The installation of most of the deep community wells took place in 2003, after the follow-up survey for a considerable number of participants, and the associated change in behavior and reduction in urinary As level may not have been fully captured. In addition, although the initial half-life of As is short (Buchet et al. 1981; Pomroy et al. 1980), the literature has documented that the human body stores substantial amounts of As (Farmer and Johnson 1990) and may excrete it in urine over a period of time, even after the exposure has ceased (Aposhian et al. 1997). Therefore, the urinary As level in persons with high exposure who switched to safe wells may not immediately respond to a drop in well As level. Together, these considerations suggest that the effectiveness of the intervention in reducing urinary As level may therefore be somewhat underestimated in the present study.

Switching to a safe well can reduce urinary As to levels almost as low as that observed in residents consuming water with $< 50 \mu\text{g/L}$. The large drop in urinary As for participants who switched from an unsafe well to a known safe well, almost to levels in the control population, is very encouraging. On the basis of these observations, we urge a revision of the governmental policy to reinforce the effectiveness of a community-based mitigation program that relies on deeper, low-As aquifers (Ahmed et al. 2006). Significant As contamination in deep aquifers is unlikely unless large

amounts of water are withdrawn for irrigation (Zheng et al. 2005). Additional governmental efforts may therefore have to be considered to manage irrigation (Ahmed et al. 2006).

Our findings not only indicate that response surveys based on interviews are reliable but they also suggest a decrease in an internal biomarker of exposure that may lead to future health benefits. Several studies have suggested that As mitigation eventually reduces As-associated morbidity. Pi et al. (2005) found that a 13-month period of consuming low-As water improved the vascular response to cold stress in Inner Mongolia, China. Another study in Chile found that provision of water with low As ($45 \mu\text{g/L}$) for 8 weeks was associated with a decrease in micronucleated cells in exfoliated bladder cells (Moore et al. 1997). On the other hand, a reduction in ischemic heart disease mortality and kidney cancer mortality was observed only decades after tap water (As free) was provided in an arseniasis-endemic area in Taiwan (Chang et al. 2004; Yang et al. 2004). We recently described a dose-response relationship between prevalence of As-related skin lesions and As exposure at baseline even at water As levels $< 50 \mu\text{g/L}$ (Ahsan et al. 2006b). The average time of exposure to baseline wells (8 years) was relatively longer than the average duration of switching wells (1.9 years). The extent to which As-related morbidity and mortality in this population is reversible by the reduction of As exposure awaits further examination with a longer follow-up of the population. The cost-effectiveness or cost-benefit issues also need to be addressed to evaluate the overall impact on the society when such data are available in the future.

Removal of As from groundwater, or human pathogens from surface water, is economically and culturally challenging, particularly on a large scale (Ahmed et al. 2006). Based on the quantitative evidence presented here, it appears that testing and monitoring of wells managed at the village level, combined with judicious installation of low-As deep community wells in high exposure areas, could rapidly reduce As exposure at the national scale.

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