XENOESTROGEN TISSUE CONCENTRATIONS CORRELATED TO BIOLOGICAL RESPONSES IN MICE

Elin M. Ulrich [1], Andrea Caperell-Grant [2], Ronald A. Hites [1], Robert M. Bigsby [2]

[1] School of Public and Environmental Affairs and Department of Chemistry, Indiana University, Bloomington, Indiana, USA 47405 [2] Department of Obstetrics-Gynecology, Indiana University School of Medicine, Indianapolis, Indiana, USA 46202

Introduction

The effects of xenoestrogens have been extensively studied in rodents and cells, but generally these studies have used very high doses (DDT *1-8*; HCH *9-13*). Using a continuous-release, low dose system in ovariectomized mice, we correlated estrogenic endpoints with concentrations in fat and blood. Silastic capsules (1, 2, or 4) containing either β-hexachlorocyclohexane (β-HCH) or *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT) were implanted subcutaneously and animals were sacrificed after 1 week. Blood and fat concentrations were determined by GC-ECD, and purity confirmed by GC-MS. Fat concentrations (ng/g) of *o,p'*-DDT and β-HCH correlated linearly to blood levels (ng/mL) (*o,p'*-DDT, $r^2 = 0.80$; β-HCH, $r^2 = 0.77$); fat was higher than blood by 74- and 150-fold for *o,p'*-DDT and β-HCH, respectively. Blood levels were 77 – 943 ng/mL for *o,p'*-DDT and 56 – 299 ng/mL for β-HCH. Vaginal epithelium thickness, ranging from an average of 10 ± 0.54 μm in controls to 114 ± 8.4 μm in treated animals, was positively correlated to blood levels of either compound (*o,p'*-DDT, $r^2 = 0.49$; β-HCH, $r^2 = 0.80$); even the lowest blood concentrations were associated with a 3- to 6-fold increased thickness. Uterine epithelial cell height ranged from 7.5 ± 0.47 μm in controls to 26.0 ± 1.73 μm in high-dose *o,p'*-DDT-treated animals ($r^2 = 0.49$). Further testing at lower doses will be required to determine the minimal blood levels associated with estrogenic activity. In this study, the lowest blood concentrations achieved from a single capsule of either compound were within 1 order of magnitude of human blood concentrations, suggesting that such levels pose a significant potential for estrogenic activity in humans.

Experimental

Mouse Treatment- Adult female ICR mice were ovariectomized, and three weeks later, groups of five animals received 1, 2, or 4 treatment capsules containing either *o,p'*-DDT or β-HCH. These capsules were made from Silastic tubing (Konigsberg Instruments, Pasadena, CA, 1.6 mm I.D., 3.2 mm O.D., and 14 mm length), sealed at each end with Silastic cement and contained 18-22 mg of material. A positive control group consisted of animals that received a Silastic capsule containing estrone (Sigma Chemical Co., St. Louis, MO), and negative control animals were treated with an empty capsule. After one week of treatment, animals were anesthetized and exsanguinated by heart puncture. The uterus and vagina of each animal was processed for histomorphometric determination of the estrogenic effect. Blood serum and intraperitoneal fat samples were analyzed for *o,p'*-DDT or β-HCH concentrations.

Histomorphometrics- Pieces of uterus and vagina were fixed in neutral formalin and processed for paraffin sections (6 μm). Tissue sections were stained with hematoxylin and eosin. Cross-sections

were examined under a light microscope (Nikon Optophot) that was interfaced with a Macintosh PowerPC computer through a Sony 3CCD color video camera. The height of the uterine epithelium and the thickness of the vaginal epithelium were determined using an image analysis program (IPLab Spectrum, Signal Analytics, Vienna, VA).

Extractions- Approximately 100 μL mouse serum diluted with 1 mL of 90% formic acid (Fisher Scientific, Fair Lawn, NJ) were extracted three times with 20 mL of hexane (EM Scientific, Gibbstown, NJ). The internal standard was γ-HCH for the mice treated with β-HCH and *p,p'*-DDT was used for the mice treated with *o,p'*-DDT (Accustandard, New Haven, CT). The resulting aqueous phase was extracted with 1 min of vortex mixing and 1 min of centrifugation for each aliquot of hexane. Fat tissues (about 0.25 g) were ground with about 15 g of 10-60 mesh anhydrous sodium sulfate (Fisher Scientific) and spiked with the appropriate internal standard as above. These mixtures were then soxhlet extracted for 24 hrs in 300 mL of 50% acetone in hexane (EM Scientific). Blanks and spikes were also extracted for quality control.

Sample Cleanup- The removal of lipids was performed using gel permeation chromatography. The glass column (2.5 cm x 100 cm) was packed with SX8 Bio-Beads (BioRad Laboratories, Hercules, CA) and eluted with 60% cyclohexane in dichloromethane (EM Scientific) at a flow rate of 10 mL/min through the column. The lipids were eluted in the first 20 min fraction, and the HCHs and DDTs were eluted in the following 40 min fraction. Sample extracts were reduced to about 1 mL by rotary evaporation and exchanged into hexane as necessary. These extracts were then run through a silica (grade 923 Grace Davison, Baltimore, MD) column consisting of glass wool, 20 cm of silica (1% HPLC grade water deactivated), and 1 cm of sodium sulfate. Three fractions were collected, with the second fraction containing the HCHs and DDTs. This fraction was rotary evaporated to 1 mL and was further reduced to about 50 μL by a stream of nitrogen and transferred into an autosampler vial with 2-3 rinses of hexane.

Instrumental Parameters- The samples were analyzed on a Hewlett Packard (HP) 5890A gas chromatograph (GC) with an electron capture detector (ECD). The carrier gas (80 mL/min) was hydrogen, and the makeup gas (25 mL/min) was nitrogen (Gas Tech, Hillside, IL). Injections were made by an autosampler in splitless mode, and the purge flow (2 mL/min) was opened after 3 min. A 60 m DB5 column (J&W Scientific, Folsom, CA) with an internal diameter of 250 μm and a film thickness of 0.1 μm was used for separation. The GC temperature programs were typical for this type of analysis; total time was 52.46 min for DDT, and 40.33 min for HCH. Quantitation was performed by relative response factors. Compound purity was determined under full scan conditions on a HP 5890 series II GC connected to a HP 5989A mass spectrometer in electron-capture negative ionization mode. Retention times for the target compounds as well as several DDT analytes were measured to ensure no compounds overlapped. The mass spectra were then compared with known compounds for purity (14).

Results and Discussion

Concentrations- The average blood concentration and standard deviation of β-HCH and *o,p'*-DDT for each treatment group are shown in each panel of Figure 1 on the left *y*-axis (triangles). As expected, the concentrations in blood and fat (data not shown) are correlated with dose. As other researchers have reported (*13, 15*), the concentration in blood and fat are linearly correlated with an r^2 of 0.77 and 0.88 for β-HCH and *o,p'*-DDT respectively (data not shown). The concentrations

in fat are higher than in the blood by a factor of 135-230 for β-HCH and 55-125 for *o,p'*-DDT. Note that the concentrations of DDT are higher than for the HCH despite being readily metabolized to DDE.

Estrogenic Response- The average and standard deviation for uterine cell height and vaginal cell thickness are shown in Figure 1 on the right *y*-axis (squares). Both β-HCH and *o,p'*-DDT produced increases in these parameters. Four capsules of DDT produced responses that were equivalent or greater than those induced by estrone. The effect of β-HCH was not as high; it is known that this compound does not bind to the estrogen receptor, but acts by some other mechanism (*9, 12*). Nishino and Neuman found that certain estrogenic compounds have much greater effects in the vagina than in the uterus (*16*). This study shows the same type of results, especially for β-HCH. Figure 1A-1D show the dose response curves for both compounds and tissues. Notice in Figure 1A that the response in the uterus exposed to β-HCH does increase, but quickly levels off. Figure 1B shows that in the vagina, the response continues to increase with dose. The *o,p'*-DDT treatments (Figure 1C and D) gave similar results in both tissues, with estrogenic responses increasing at all doses.

It is important to note that the concentrations of xenoestrogens in this study are very close to the levels of contaminants in humans. Concentrations of β-HCH in human blood range from 0.13 to 18 ng/mL (*17-18*) and in fat from <1 ng/g to 840 ng/g (*15, 18*). These values are either within an order of magnitude or exceed the concentrations in this study. A group of Israeli men had blood concentrations of *o,p'*-DDT of 5.8 ng/mL (*18*), and a Canadian study found blood levels up to 0.27 ng/mL and fat levels up to 9.1 ng/g (*17*). While *o,p'*-DDT concentrations in this study are much higher, the estrogenic response is also quite high. Future work includes using lower doses to determine the no effect levels for these compounds. We expect those levels to be close to the range of human exposure considering the effects shown here.

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Figure 1. Concentration curves (triangles) on the left axis (β-HCH A/B, *o,p'*-DDT C/D) and estrogenic response curves (squares) on the right axis (uterus A/C, vagina B/D) in treated mice.