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Report

The link between the insecticide heptachlor epoxide, estradiol, and breast cancer

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Summary

Given the suspected effects of estrogens on breast cancer, xenoestrogenic insecticides may be a risk factor. Studies of the weak xenoestrogen, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), have failed to demonstrate a causal relationship, though another estrogenic organochlorine insecticide, dieldrin, belonging to the cyclodiene family, has recently been linked to breast cancer. Other cyclodienes such as heptachlor epoxide (HE) and oxychlordane (OC) present in breast tissue have not been evaluated as rigorously, presumably due to their lower concentration and lower recovery using solvent extraction procedures. We used sparging extraction coupled with gas chromatography to determine the levels of HE, OC, and DDE in adipose tissue within breast biopsies in a series of 34 women evaluated for breast abnormality. Of the three insecticides tested, only HE ($p = 0.007$) was positively associated with prevalence of breast cancer in the biopsies. In rapid, non-genomic studies using isolated human leukocytes, flow cytometric methods were used to measure HE-induced oxidants and DNA damage. These studies indicated that HE, at concentrations similar to those in breast biopsies, induced an inverted-U increase in intracellular oxidants and DNA strand breaks [both blocked by specific nitric oxide- (NO-) synthesis blockade with L-NMMA] in human polymorphonuclear leukocytes (PMNs). HE-treated PMNs also induced damage to surrounding lymphocytes in mixed-leukocyte incubations (also inhibited by NO blockade). The HE-induced changes in NO were inhibited by 17β-estradiol-(17β-E_2) receptor antagonists and were mimicked by similar concentrations of 17β-E_2. The addition of tumor necrosis factor-α (TNF-α) increased intracellular oxidants and DNA damage and shifted the responses to lower HE concentrations. This study, along with others, suggests that HE-induced NO production may contribute to initiation, promotion, and progression of cancer.

Introduction

One in eight women in the United States will develop breast cancer [1]. Breast cancer is the second leading cause of mortality among women in the United States, with 184,300 new cases and 44,300 deaths in 1996 [2]. Breast cancer rates in the United States are among the highest in the world, with rates 3–7 times those found in Asia. Numerous studies have shown that when Asian women migrate to the United States their risk of breast cancer increases over generations approaching that of United States Caucasians, suggesting that environmental/lifestyle factors in the United States play a substantial role in the etiology of breast cancer [3]. The incidence of breast cancer in the United States increased 36% from 1973 to 1987, and since 1987 has remained relatively constant [4]. However, the mortality related to breast cancer decreased 12% between 1990 and 1999. The increase in mammography use for early detection of breast cancer has been cited as a contributor to the increased incidences in breast cancer between 1973 and 1987, but cannot account for the total ascent [5]. Given that estrogen therapies [6, 7] and higher serum levels of estradiol in postmenopausal women [8, 9] have been linked to breast cancer, it has been proposed that xenoestrogenic compounds might be a risk factor for the disease [10].

Recently, serum dieldrin, an organochlorine compound belonging to a class of insecticides called cyclodienes, has been reported as a risk factor for breast cancer in Denmark [11]. Cyclodienes (chlordane, heptachlor, and aldrin) were introduced in the United States in the mid '50s. Usage peaked in the late '60s, and they were banned for agricultural use in 1978 [12]. In humans and domesticated animals, these cyclodienes are metabolized to their respective epoxides, oxychlordane (OC), heptachlor epoxide (HE), and dieldrin that accumulate in lipid-containing compartments [13–15]. Serum levels of HE and OC have declined little in humans since their agricultural uses were banned, probably reflecting the continual inhalation of chlordane and heptachlor by an estimated 50 million persons living in 30 million homes treated for termites in the United States between the mid-'50s and 1988 [12, 16–18]. The increase in heptachlor exposure by the inhalation route corresponds to the increased incidence in breast cancer from 1973 to

1987. However, evaluation of inhalation exposures in animal models for breast cancer has not been performed. Compared to cyclodienes, agricultural use of another estrogenic insecticide 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) peaked a decade earlier and residue levels of its main metabolite, DDE, declined markedly in meat, dairy products, and human adipose tissues during the '70s and '80s [12].

Only four studies have evaluated the relationship between fat levels of HE or OC in biopsies and breast cancer. Of the four studies, two assessed only HE [19, 20], one assessed only OC [21], and the fourth assessed the sum of the values for HE and OC [22]. With the exception of the study that assessed only OC, these studies were small, with 5–12 breast cancer patients. All four studies used solvent extraction techniques, followed by cleanup procedures that may have resulted in the loss of these semi-volatile insecticides. The vapor pressure of HE is approximately 1000 times that of DDE. To study the relationship of these insecticides in breast tissue and the prevalence of breast cancer, we eluted the insecticides from the biopsy fat by sparging [23]. Sparging increases the extraction efficiency and minimizes the loss of HE and OC during intermediate cleanup and concentration steps.

Heptachlor and chlordane are reported to be estrogenic in a variety of models [23–28]. However, the estrogenic potential of their epoxides (HE and OC), at concentrations found in the body, have not been studied in isolated human cells. We have shown that HE increases oxidant levels that damage DNA and cell membranes by a 17β-E_2 receptor-mediated process. We propose that HE-induced DNA damage may contribute to breast cancer in exposed women.

Materials and methods

Insecticides in breast biopsies–prospective clinical study

Patients and design

A fatty breast tissue sample was obtained from each of 34 women seen for initial surgical evaluation of a palpable breast mass or mammographic abnormality at Brooke Army Medical Center (San Antonio, Texas) from 1994 to 1998. The selection criteria were grossly unremarkable fatty breast tissue (not required for pathological examination) present on the breast biopsy, patient-signed informed consent for measuring insecticides in the adipose specimen, and patient questionnaire participation. This prospective study was approved by the Brooke Army Medical Center Institutional Review Board. The adipose samples were coded and frozen at −75 °C in small, airtight, screw-cap vials, until analysis for insecticides, which was performed without knowledge of the biopsy results. The pre-biopsy questionnaire requested responses regarding risk factors for breast cancer. The outcome variable was malignancy in the breast tissue of the biopsy.

Histopathology

Histologic sections were prepared from patients whose fatty breast tissue was submitted for insecticide evaluation. These sections were prepared from formalin-fixed, paraffin-embedded tissue, stained with hematoxylin-eosin. All cases were examined by one of the authors (SN) without knowledge of tissue insecticide results. Benign breast diseases were identified by morphologic features, such as intraductal hyperplasia, atypical intraductal hyperplasia, atypical lobular hyperplasia, lobular neoplasia, and sclerosing adenosis which were documented in each histopathology report. Carcinomas were identified and classified according to the 1989 TNM classification; and tumor grade, where appropriate, was established using a combined architectural and cytologic grading system for infiltrating ductal carcinoma [29].

Insecticide measurements

HE, OC, and DDE in breast-biopsy fat were extracted by sparging and analyzed by gas chromatography [23]. Briefly, a Dynamic Thermal Stripper (Environchem, Kemblesville, Pennsylvania) was used to sparge the insecticides from 5 mg of adipose tissue that had been homogenized (1 min) and sonicated (1 min), and then placed in 10 ml of distilled water, along with 500 pg of trichlorobiphenyl used as a control to demonstrate consistent extraction recoveries. Fat homogenates were sparged twice with ultra-pure nitrogen for 30 min at 120 °C trapping the insecticides on Tenax solid sorbent. After each sparging, the Tenax solid sorbent tube containing the insecticides was subsequently thermal-desorbed by use of an Unacon 810 Inletting System (Environchem, Kemblesville, Pennsylvania) into a SPB-608 30 M, fused silica capillary column (J & W Scientific, Folsom, California) of a HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, California) equipped with an electron capture detector. Compared to solvent extraction procedures, sparging eliminates cleanup and concentration steps where semi-volatile analytes may be lost and allows the entire extract to be eluted directly into the capillary column. The amount of each insecticide in each biopsy sample was determined by adding the results of first and second sparging/analysis and comparing responses to standard curves constructed from certified standards (Ultra Scientific, Kingstown, Rhode Island). The standard curves were generated by spiking known concentrations of HE, OC and DDE into 5 mg of homogenated breast biopsy fat derived from three individual specimens previously shown to have low levels of insecticides. Insecticide levels in the unspiked composite were subtracted from each spiked composite. The coefficient of variation for spiked trichlorobiphenyl into biopsy samples was between 10–15%. The coefficient of variation for HE, OC, and DDE in a composite of three biopsies having median levels of insecticides was 10, 16 and 20%, respectively. HE, OC, and DDE concentrations were expressed as ng/g wet weight of tissue.

In-vitro studies

Cell-donor subjects and general methods and design

In-vitro studies were conducted with isolated leukocytes derived from blood donated by healthy, non-smoking men who had previously given informed consent. Since we wanted to study the effects of estrogenic compounds, male donors were used to provide leukocytes with low prior exposure to estrogens. These studies were approved by the US Army Institute of Surgical Research Human Use Committee.

Polymorphonuclear leukocytes (PMNs) and lymphocytes were obtained from EDTA-preserved venous blood by layering blood over Polymorphprep (Nycomed Pharma, Oslo, Norway) followed by centrifugation as previously described [30]. The purity of PMNs and lymphocytes was greater than 88 and 95%, respectively, and their viability, as determined by trypan-blue exclusion, was greater than 90%. All experiments, except mixed PMN/lymphocyte studies, were conducted with 300,000 PMNs added to 100 μl of Hanks Balanced Salt Solution (HBSS) and incubated with Ca^{2+} (50 μM) and physiological levels of arginine (100 μM). Replicate experiments were conducted using PMNs or PMNs and lymphocytes isolated from different donors.

Incubations were designed to assess the effect of a series of HE concentrations without and with additional influence from blockade of NO production or of 17β-E_2-receptor binding, or the presence of TNFα on PMN intracellular oxidant level and DNA strand breaks. In a similar set of incubations, the effect of a series of 17β-E_2 (instead of HE) concentrations on oxidants was assessed without and with blockade of NO or 17β-E_2. In addition, with mixed-leukocyte incubations, the effect of varying concentrations of PMNs (not treated or treated with one concentration of HE without or with NO blockade) on lymphocyte cell-membrane integrity was assessed. These three endpoints: oxidants, DNA damage, and cell-membrane integrity, were determined by flow cytometric measurements of fluorescent probes. For each endpoint, fluorescent intensities were normalized by dividing the fluorescence from treated leukocytes by that from untreated leukocytes (control value) from the same donor.

Effect of HE (or 17β-E_2) on intracellular oxidants

Intracellular levels of oxidants in PMNs were measured using a previously reported flow cytometric procedure [30]. Briefly, PMNs were loaded with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA)(Kodak, Rochester, New York) at 37 °C for 15 min in a gently rocking water bath followed by a 10 min incubation without and with the specific NO synthase inhibitor N-methyl-L-arginine (L-NMMA) (5 mM) (Calbiochem, San Diego, California) [30], 17β-E_2 receptor antagonists [tamoxifen (200 nM) (Sigma, St. Louis, Missouri) or ICI 182,780 (0.1 nM) (a gift from Zeneca Pharmaceuticals, Cheshire, England)], or TNFα (10 ng/ml) (Sigma, St. Louis, Missouri). Tubes containing DCFH-loaded PMNs without and with inhibitors or TNFα were subsequently incubated with varying concentrations of HE (zero and 0.01–10 nM) (Ultra Scientific, Kingstown, Rhode Island) at 37 °C for 40 min. Flow-cytometric analyses of oxidized DCFH in PMNs were performed with an argon laser (488 nm) and emission light measured behind a filter transmitting 530/30 nm light on a FACScan, Becton Dickinson (San Jose, CA) with CELLQuest data acquisition and analysis software. For each sample, 10,000 PMNs were analyzed. The mean channel fluorescence was determined on a linear scale from a single parameter histogram. In a second set of incubations, varying concentrations of 17β-E_2 (zero and 0.01–10 nM) were used instead of varying concentrations of HE. Other elements were the same as described for incubations with HE, except that incubations with ICI 182,780 and TNFα were not included (though incubations with L-NMMA or tamoxifen were included). Data normalization after both sets of incubations is described in the previous section.

Effect of HE on DNA strand breaks

HE-induced DNA strand breaks in PMNs were measured by flow cytometry using the terminal deoxynucleotidyl transferase-mediated nick end-labeling assay (TUNEL) [30] [*In-Situ* Cell Death Detection Kit (Boehringer Mannheim, Indianapolis, Indiana) according to manufacturer's instructions]. PMNs were incubated at 37 °C with and without the previously-mentioned concentrations of L-NMMA, 17β-E_2 receptor antagonists, or TNFα, and varying concentrations of HE as described above for the HE-induced DCFH oxidation incubations, with the exception that HE incubation time was 2 h, instead of 40 min. Fluorescence was measured by flow cytometry using the parameters as described above. Data normalization was also described above.

Effect of HE-treated PMNs on cell-membrane integrity of co-incubated lymphocytes

The effect of oxidants, derived from HE-treated PMNs, on the membrane integrity of neighboring lymphocyte target cells was determined by propidium iodide (PI) exclusion in a mixed PMN/lymphocyte preparation using 100,000 lymphocytes and PMN/lymphocyte ratios of 0/1, 2/1, 4/1, and 10/1) [30]. To obtain these ratios, varying numbers of PMNs (prepared at a density of 300,000/100 μl HBSS) from four different treatment preparations were mixed with lymphocytes (100,000/100 μl HBSS) from the same donor and incubated for 40 min at 37 °C. The four treatment preparations were: (1) untreated PMNs (control series); (2) PMNs treated with HE (1 nM) for 1 min; (3) and (4) PMNs preincubated for 10 min with either L-NMMA (5 mM) or 2-(4-carboxyphenyl)-4,4,5, 5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) (5 mM) (Calbiochem, LaJolla, California), a NO scavenger, followed by a 1-min incubation with HE (1 nM). At the end of the 40-min incubation, PI (0.75 mM) was added for 2–3 min before determining the PI content of the lymphocytes by

flow cytometric procedures. Lymphocytes having a normal intracellular PI level (at or below the PI fluorescence cut point characteristic of normal lymphocytes incubated without PMNs or HE) were scored as undamaged. Lymphocytes with PI above the normal limit were scored as damaged. To normalize the data, the fraction of undamaged lymphocytes in the each sample (incubation) was divided by the subject-specific fraction of undamaged lymphocytes from those control-series samples which received no PMNs (ratio 0/1 PMNs/lymphocytes).

Data analysis

Data were analyzed with SPSS software (Chicago, IL). Test p values <0.05 were considered statistically significant. Univariate and *post-hoc* comparisons were two-tailed.

Insecticides in breast biopsies

Univariate comparisons between patient-outcome groups (benign versus malignant biopsy result) with regard to age, body weight, height, body mass index in weight per height squared (kg/M^2), age at menarche, number of full-term pregnancies, cumulative breast-feeding time, breast adipose organochlorine concentrations, history of oral contraceptive use, history of estrogen replacement therapy, and family history of breast cancer were made by χ^2 test (the last three) or by Mann–Whitney U test (the others). Binary multiple logistic regression on the biopsy result was performed with likelihood-ratio convergence and backward stepping, in order to assess independent contribution of each covariate mentioned above, while accounting for any contribution of the others. Any covariate with a significant ($p < 0.05$) contribution to the logistic model remained in the final logistic equation. In addition, this logistic equation was used to calculate the predicted probability of malignancy in the biopsy of each patient. For this purpose, any covariate in the equation, other than HE, was set at its respective overall observed median value in order to provide a probability of malignancy related to HE but adjusted to a common value for any other significant covariate. Patients were then divided into quartiles (Q1–Q4) of observed tissue HE. The median logistic probability of malignancy in each Q of observed HE was divided by that in Q1 as the reference, in order to obtain adjusted relative risk of malignancy in each HE quartile for graphic display [31].

In-vitro effect of HE (or 17β-E_2) on intracellular oxidants or DNA strand breaks

We used one-way ANOVA across HE (or 17β-E_2) concentrations or, if variance equality (Levene test) was rejected, Kruskall–Wallis tests instead of ANOVA. Comparisons between overall mean values (for those samples receiving PMNs) between two incubation series and an accounting for multiplicity of such comparisons were made by Tukey HSD tests or, if variance equality was rejected, Tamhane tests. If a change in oxidants or DNA breaks across HE or 17β-E_2 concentrations was detected by ANOVA or a Kruskall–Wallis test, then HE (or 17β-E_2) concentrations were log-transformed and the data were fitted to a standard Gaussian model [$y = a + b * \exp(-.5 * ((x-c)/d)^2)$] with SPSS non-linear regression to estimate the parameters (a, b, c, and d) and their error terms [where y = response variable, x = HE or 17β-E_2 concentration as log M, a = baseline response (BL), b = response amplitude (AMP, maximum response minus BL), c = log concentration at maximal response (log CMR) in log M units, and d = curve width factor (WF) also in log M]. A Gaussian curve was used to approximate the inverted-U shape of a given response to various concentrations of HE or 17β-E_2. A significant r^2 for a model indicated that the data fit a Gaussian rise and fall better than a non-fluctuating horizontal line at the overall mean response. Comparison of Gaussian parameters between two treatment series was made by testing the significance of a different grouping parameter which, when multiplied by the grouping code, was added to each respective original Gaussian parameter (SPSS non-linear regression).

Effect of HE-treated PMNs on cell-membrane integrity of co-incubated lymphocytes

Lymphocyte cell-membrane integrity, as altered by HE-treated PMNs without and with NO inhibitors, was analyzed by two-way ANOVA. Lymphocyte membrane integrity with PMNs present (data were donor-specific normalized response means with PMN/lymphocyte ratio > 0/1) was compared among treatments by a Tukey HSD test.

Results

Insecticides in breast biopsies

Table 1 shows the distribution of the demographic and historic covariates, together with tissue concentrations of HE, OC, and DDE, between the two groups defined by a benign or malignant biopsy result. Only age ($p = 0.006$) and HE ($p = 0.01$) differed significantly between groups. In addition, multiple logistic regression detected only age ($p = 0.004$) and HE ($p = 0.007$) for association with malignancy in the breast biopsy, both positively correlated with the likelihood of malignancy (probability of malignancy = $1/(1+\exp(-\ln(\text{odds}))$; where $\ln(\text{odds}) = -8.56 + 0.185 * \text{HE} + 0.113 * \text{age}$, and biopsy malignancy was coded as 0 for "no" or 1 for "yes"). The median values of HE for the HE quartiles (Q) were 3.6, 5.9, 10.7, and 15.9 ng/g, respectively, varying 4.4-fold from Q1 to Q4. The observed proportions of patients with malignancy in Q1–Q4 were, respectively, $2/9 = 22.2\%$, $3/8 = 37.5\%$, $5/8 = 62.5\%$, and $7/9 = 77.8\%$. The respective age-adjusted median risks were similar, 23.7, 32.4, 53.5, and 75.1%. The age-adjusted median risk in Q4 was 3.2-fold that in Q1 (95% confidence interval 1.1- to

Table 1. Univariate analyses

Continuous variables	Breast Biopsy Group				Mann-Whitney U Test (two-tailed p)
	Benign (n = 17)		Malignant (n = 17)		
	Median	Range	Median	Range	
Age (years)	53	35–77	62	51–82	0.0058
Body weight (pounds)	150	111–242	138	92–252	NS
Height (inches)	64	60–71	64	58–68	NS
Body mass index (kg/M^2)	25.7	20.3–43.0	23.7	19.3–44.7	NS
Menarche (age, years)	13.0	9.0–15.0	12.5	11.0–16.0	NS
Number of full-term pregnancies	2.0	0.0–4.0	3.0	0.0–6.0	NS
Breast feeding (years, cumulative)	0.0	0.0–2.3	0.0	0.0–3.0	NS
Breast adipose heptachlor epoxide (HE, ng/g)	5.6	0.7–15.9	12.5	3.3–123.3	0.0106
Breast adipose oxychlordane (OC, ng/g)	37.1	16.0–87.0	51.1	24.6–300.5	NS
Breast adipose (DDE, ng/g)	949	358–3356	975	360–4107	NS
	Benign (n = 17)		Malignant (n = 17)		χ^2 Test (p)
Categorical variables	n	%	n	%	
Oral contraceptive use					
no	6	35.3	11	64.7	NS
yes	11	68.8	5	31.3	
Estrogen replacement therapy					
never	8	53.3	7	46.7	NS
past	1	20.0	4	80.0	
current	8	61.5	5	38.5	
Family history of breast cancer					
no	11	55.0	9	45.0	NS
yes	6	46.2	7	53.8	

9.2-fold) with a steady progression of relative risk from Q1 to Q4 (Figure 1). In this set of 34 patients, we detected only age and breast-adipose-tissue HE as independent determinants of breast cancer.

In-vitro studies

Effect of HE (or 17β-E_2) on intracellular oxidants and of HE on DNA strand breaks

Incubation of PMNs for 40 min with HE concentrations (0.01–10 nM) produced an inverted-U-shaped response in the mean concentration of intracellular oxidants ($p < 0.001$, ANOVA and Gaussian fit) and DNA strand breaks ($p < 0.05$, ANOVA; $p < 0.001$, Gaussian fit) (Figure 2A and C, Table 2A and C). Similarly, 17β-E_2 (also tested over 0.01–10 nM) increased the overall concentration of oxidants ($p < 0.01$, ANOVA; $p < 0.001$, Gaussian fit) (Figure 2B, Table 2B), peaking at a 17β-E_2 concentration (about 0.6 nM) (equates to CMR of $10^{-9.3}$ M) similar to that for HE (about 1 nM) (CMR of $10^{-8.9}$ M). The peak in DNA strand breaks occurred at approximately 3–4 nM HE (CMR of $10^{-8.5}$ M) (Figure 2C, Table 2C). Prior incubation of PMNs for 10 min with a specific NO synthase inhibitor (L-NMMA) or a 17β-E_2 receptor inhibitor (ICI 182,780 or tamoxifen) eliminated the inverted-U response induced by HE on oxidant concentrations (Figure 2A, Table 2A) and DNA strand breaks (Figure 2C, Table 2C). Likewise, incubation of PMNs for 10 min with L-NMMA or tamoxifen prior to 17β-E_2 treatment resulted in the elimination of the inverted-U effect on intracellular oxidants (Figure 2B, Table 2B). These results suggested that HE was interacting with a 17β E_2-like receptor increasing the oxidant NO which induced DNA damage. Incubation of PMNs with the inflammatory cytokine TNFα (10 ng/ml) for 10 min prior to HE exposure increased the overall intracellular oxidant levels by 37% ($p < 0.001$) and DNA strand breaks by 109% ($p < 0.05$) (Figure 2A and C, Table 2A and C). In addition, TNFα lowered the HE concentration at maximal response (CMR) for oxidants approximately from 1 nM HE (without TNFα) to 0.1 nM HE (CMR of $10^{-10.2}$ M) (with TNFα) ($p < 0.001$, Figure 2A, Table 2A). TNFα also lowered the HE CMR for DNA strand breaks approximately from 3 nM HE (without TNFα) to 0.3 nM HE (CMR of $10^{-9.6}$ M) (with TNFα) ($p < 0.01$, Figure 2C, Table 2C).

Effect of HE-treated PMNs on cell-membrane integrity of co-incubated lymphocytes

Lymphocytes, used as target cells, were co- incubated for 40 min with varying ratios of PMNs treated without and with HE (1 nM) following pre-treatment with either an inhibitor of NO production (L-NMMA) or a scavenger of NO (carboxy-PTIO). Lymphocyte membrane integrity decreased with increasing PMN/lymphocyte ratios ($p < 0.001$) (two-way ANOVA) (Figure 3). Cell-membrane integrity of lymphocytes incubated with HE-treated PMNs was lower (Tukey HSD test) than that in incubations with each of the following: (1) untreated PMNs ($p < 0.001$), (2) HE-treated PMNs previously loaded with L-NMMA ($p < 0.05$), and (3) HE-treated PMNs previously loaded with carboxy-PTIO ($p < 0.001$).

Discussion

We measured the levels of three organochlorine insecticides (HE, OC, and DDE) known to cause cancer in laboratory animals and to have weak estrogenic effects on gene expression or binding to nuclear estrogen receptors [24, 32, 33]. Of the three, only concentrations of HE in breast biopsy fat was linked to cancer. These results are consistent with previous feeding studies in rodents indicating that HE and heptachlor are more potent liver carcinogens than chlordane with heptachlor epoxide residing in the most potent quartile of suspected carcinogens [32]. However, studies of the cancer risk resulting from breathing these compounds have not been performed.

Of the previously-reported risk factors analyzed in this study, only age was detected as a predictor of breast cancer, probably because other previously-reported risk factors require a greater number of patients for detection. HE in adipose tissue has been reported to increase with age [18] and may contribute to the age-related risk of breast cancer. HE is linked to other risk factors such as body mass index. HE, like other lipophilic organochlorine insecticides, accumulates in the food chain with the highest concentrations found in the fat of meat and dairy products. A high fat diet has been shown to result in a higher body mass index and elevated levels of HE in serum [34], whereas a vegetarian diet results in lower cyclodiene concentrations in the fat of breast milk [35, 36]. Lactation, a suspected negative risk factor, recently has been reported to decrease the relative risk of breast cancer by 4.3% for every 12 months of breast-feeding [37]. Since lactation has been reported to be the most important route for reducing body burden levels of organochlorine compounds [22, 38], the levels of HE and related compounds in breast tissues would decrease in exposed mothers during lactation [39, 40].

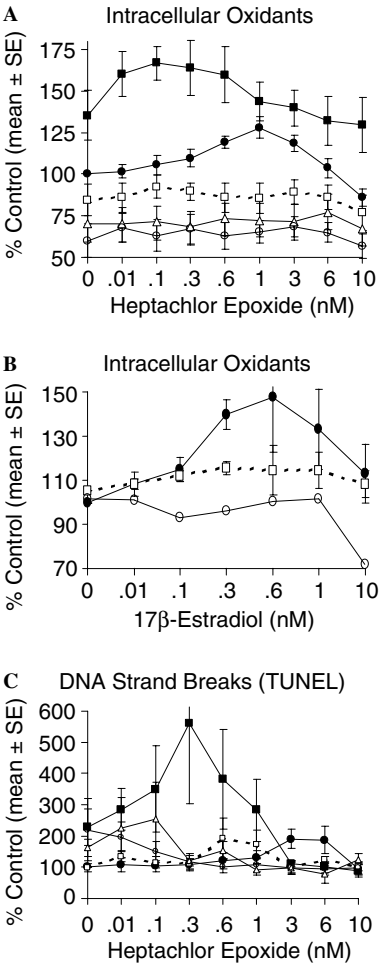

Figure 2. HE- and 17β-E_2-induced intracellular levels of oxidants and DNA strand breaks in human PMN. Levels of intracellular oxidants induced by various concentrations of HE (●) (A) or 17β-E_2 (●) (B), and levels of DNA strand breaks induced by HE (●) (C) in PMN without and with pre-treatment with L-NMMA (□), tamoxifen (○), ICI 182,780 (△), or TNF-α (■). Data are expressed as percent of the value of the same donor's untreated PMN value and depicted as mean ± SE. (For analysis of comparisons, see Table 2).

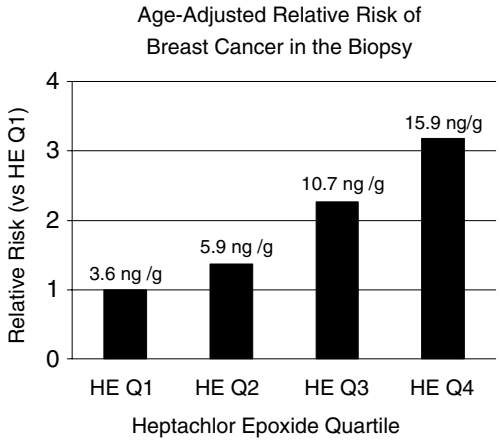

Figure 1. Progression of age-adjusted risk of malignancy in the breast biopsy over the quartiles of observed breast adipose heptachlor epoxide (HE) concentration. The 95% confidence limits on relative risk for the fourth quartile (Q4) are 1.1 and 9.2. Multiple logistic regression detected simultaneous independent contributions to the probability of malignancy from both age ($p < 0.004$) and breast adipose HE ($p < 0.007$). Median concentration of HE for each quartile is entered above the bars.

Table 2. Incubation of normal human PMNs with estrogenic compounds (See Figure 2A, B, and C)

(A) Intracellular oxidants (NO activity) at 9 concentrations (0–10 nM) of heptachlor epoxide (HE)

In-Vitro treatment	PMN[#] donors	Overall mean (%)[†‖]	One-Way ANOVA[‡]	Gaussian Parameters[‡§]				Gaussian fit r^2 [‡]
				BL[‖]	AMP[‖]	logCMR	WF	
HE	6	108.2	***	99	30.6	−8.9	0.37	0.392***
HE + TNF[¶]	6	148.0***	*	121	49.0	−10.2***	1.17	0.128**
HE + NMMA[††]	6	86.2***	NS					
HE + ICI[‡‡]	5	71.2***	NS					
HE + TFXN[§§]	3	64.0***	NS					

(B) Intracellular oxidants (NO activity) at 7 concentrations (0–10 nM) of 17β-estradiol (17β-E_2)

In-Vitro treatment	PMN donors	Overall mean (%)[†‖]	Kruskall–Wallis[‡]	Gaussian Parameters[§]				Gaussian fit r^2 [‡]
				BL[‖]	AMP[‖]	logCMR	WF	
E_2	5	120.4	**	107	39.8	−9.3	0.36	0.283***
E_2 + NMMA	5	110.9	NS					
E_2 + TFXN	1	94.1***	NS					

(C). DNA strand breaks (TUNEL) at 9 concentrations (0–10 μM) of heptachlor epoxide (HE)

In-Vitro treatment	PMN donors	Overall mean (%)[†]	Kruskall Wallis[‡]	Gaussian Parameters[§]				Gaussian fit r^2 [‡] .
				BL[‖]	AMP[‖]	logCMR	WF	
HE	5	127.0	*	104	96	−8.5	0.30	0.277***
HE + TNF	5	265.5*	***	130	428*	−9.6**	0.37	0.217**
HE + NMMA	5	133.4	NS					
HE + ICI	4	145.3	NS					
HE + TFXN	4	131.2	NS					

[#]PMN donors: number of individuals donating PMNs for all treatments and concentrations.
[†]Comparisons (Overall mean and Gaussian parameters) are versus HE (or 17β-E_2) alone (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
[‖]Overall mean, BL, and AMP are given as percent of donor's control values (no treatment) for a given panel (A, B, or C) of *in-vitro* treatments.
[‡] Fluctuation of response over HE (or 17β-E_2) concentrations (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
[§]Gaussian parameters: BL, baseline; AMP, amplitude (maximal response minus BL); logCMR, log concentration (log M) at maximal response; WF, width factor (log M). Log M refers to HE (panels A, C) or 17β-E_2 (panel B).
[¶]TNF: tumor necrosis factor alpha.
††NMMA: N-methyl-L-arginine.
[‡‡]ICI: ICI 182780.
§§TXFN: tamoxifen.

In this study, the median concentration of HE in the quartile with the highest concentrations (15.9 ng/g) (Q4) was more than four times that of the lowest quartile (3.6 ng/g) (Q1) with the age-adjusted median risk in Q4 3.2-fold that of Q1 (95% confidence interval 1.1- to 9.2-fold). The median tissue concentration of HE in the highest quartile is approximately 50 times the level of 17β-E_2 in postmenopausal (0.29 ng/g) [41] and 30 times that of premenopausal (0.5 ng/g) women [42]. Since the molecular weight of 17β-E_2 (272 g/mole) is approximately 70% that of HE (388 g/mole), the median value of HE in the highest quartile would translate to about 38 and 22 molecules of HE for each molecule of 17β-E_2 found in breast tissue of normal post- and pre-menopausal women, respectively.

Even though HE concentrations in breast tissue of this study were higher than the reported levels for 17β-E_2, HE concentrations were still several orders of magnitude lower than required to bind to nuclear 17β-E_2 receptors [32] or to induce gene transcription [33]. Furthermore, in line with the consideration that neither heptachlor nor heptachlor epoxide has been found to affect genomic endpoints, [43, 44] a pathway for HE effects on cancer development has not been described heretofore.

In order to address how environmentally- relevant levels of heptachlor epoxide may lead to breast cancer, we used a different approach and assessed a rapid, non-genomic pathway. Effects of HE at concentrations ranging from 10 pM to 10 nM were determined with

respect to oxidant production and cellular damage. Furthermore, since several investigators [45] have reported estrogen-induced concentration-dependent biphasic (inverted U) responses in a variety of endpoints, the responses for the series of HE (or 17β-E_2) concentrations in the isolated-leukocyte studies were analyzed for fits to a Gaussian-shaped curve.

Our comparisons of HE and 17β-E_2 on a molecular basis for the rapid production of oxidants (within 40 min) in a human PMN model revealed that both compounds produced similar inverted U-shaped (Gaussian-like) response curves with the concentration of HE (1.26 nM) required to induce peak production of oxidants similar to that of 17β-E_2 (0.5 nM). Pre-incubation of HE- or 17β-E_2-treated PMNs with a specific NO synthase inhibitor (L-NMMA) [30] or 17β-E_2 receptor inhibitors (ICI 182,780 or tamoxifen) suggested that NO was the oxidant produced and that it was produced by a 17β-E_2 receptor-mediated pathway. In similar studies using an amperometric probe to measure NO levels in human PMNs or monocytes, 17β-E_2 or 17β-E_2 conjugated with bovine serum albumin (both 1 nM) induced peak levels of intracellular Ca^{2+} and NO within 4 min of treatment and could be blocked by tamoxifen or ICI 182,780 (at least in moncytes) [46, 47]. Similar induction of NO by either17β-E_2 or its impeded conjugate, 17β-E_2-BSA, suggested that the receptor was located in the cell membrane [47].

One pathway by which 17β-E_2 increases constitutive NO synthase activity in a variety of cell types is by binding to a cell membrane receptor resulting in the phosphorylation of serine 1177 of NO synthase [48]. Recent results indicate that 17β-E_2-induced NO synthase phosphorylation is mediated by mitogen-activated protein kinase and/or protein kinase B (Akt) that can be blocked by the 17β-E_2 receptor antagonist ICI 278,780 [49, 50]. As compared to nuclear receptors, membrane binding sites for 17β-E_2 may have different ligand binding properties [51]. For example, a truncated variant of ERα (ER46) found in the cell membrane has been shown to be more efficient than the untruncated ER at inducing cNO synthase phosphorylation, suggesting the possibility that these ER variants may have a high affinity for xenostrogens such as HE [52]. Accordingly, bisphenol-A, previously described as a weak xenoestrogen (1000-fold less potent than 17β-E_2 for binding with the nuclear estrogen receptors), has been reported to induce rapid Ca^{2+} signaling at concentration similar to 17β-E_2 (1 nM) by binding to an estrogen receptor located in the cell membrane unrelated to ERα or ERβ [53].

Recently, heptachlor has been reported to induce the activity of protein kinase C and MAP kinases in rat hepatocytes within 45 min of treatment suggesting a non-genomic pathway [44]. The receptor that mediated the activation of these kinases was not described. However, it was shown that an environmentally relevant concentration of heptachlor of 1 nM doubled the activity of the membrane-bound protein kinase C. This concentration of heptachlor is 1000-fold lower than the concentration required to induce genomic effects [24, 33]. This low concentration of heptachlor also decreased an apoptotic marker and increased mitogenic activity within 24–48 h after treatment in rat hepatocytes, suggesting that the rapid non-genomic effects may result in long-term cellular alterations that increase the risk of cancer.

In this study, HE increased DNA strand breaks in PMNs by an apparent 17β-E_2 receptor- and a NO-mediated pathway with a concentration of 3 nM producing peak DNA damage. NO previously has been reported to cause DNA damage and mutation in human cells [54] which may lead to cancer [55]. When HE-treated PMNs were incubated with relevant levels of TNFα (10 ng/ml), not only did TNFα increase the overall intracellular oxidant levels and levels of DNA strand breaks, but it also shifted the inverted U response curves an order of magnitude to the left (10-fold lower concentration) for peak NO levels and for DNA strand breaks. These results suggest that low levels (10 ng/ml) of the inflammatory cytokine, TNFα, possibly derived from infiltrating leukocytes or resident macrophages, may act synergistically with estrogenic compounds to induce NO-mediated DNA damage and initiate neoplastic transformations. Our mixed PMN/lymphocyte studies demonstrate that NO derived from HE-treated PMNs can damage neighboring cells. The idea that inflammation may contribute to breast cancer is supported by the observation that women with chronic mastitis have an approximate 2.5-fold increased risk of developing breast cancer [56]. Since NO synthase has been found in epithelial cells [57], endothelial cells, and macrophages of breast cancers [58, 59] and since NO production in T47D breast cancer cells can be induced by 17β-E_2 [60], elevated levels of NO in breast tissue

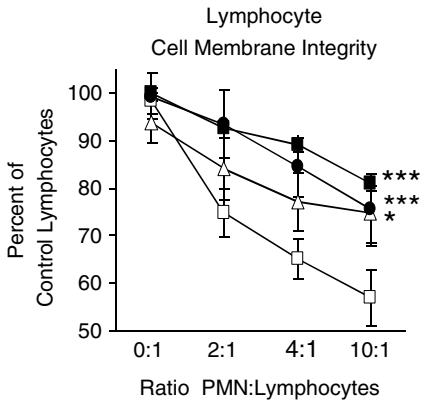

Figure 3. HE-induced oxidants produced by human PMNs damages cell membranes of neighboring lymphocytes. Membrane integrity of lymphocytes incubated with varying ratios of HE-treated PMNs (□) was lower than that of lymphocytes incubated with PMNs without HE treatment (control series) (■) (***$p < 0.001$), HE-treated PMNs pretreated with L-NMMA (△) (*$p < 0.05$), or HE-treated PMNs pretreated with carboxy-PTIO (●) (***$p < 0.001$). Data are expressed as a percent of control (i.e., the fraction of undamaged lymphocytes in each incubation divided by the fraction of undamaged lymphocytes from the same donor in the incubation without PMN from the control series) and depicted as means ± SE, $n = 6$.

may result from the chronic activation of a variety of cell types by xenoestrogens like HE.

Results of this study link HE levels in breast tissue to breast cancer. Low nanomolar concentrations of HE found in fat of breast biopsies induce rapid NO production and DNA damage through an apparent 17β-E_2 receptor-mediated pathway in human PMNs at approximately the same potency as 17β-E_2. These estrogenic-mediated effects are augmented by relevant levels of an inflammatory cytokine (TNFα). This model may have considerable relevancy, in that NO synthase activity (NO production) in breast tumors correlates with the grade of the tumors [57] and that transfecting NO synthase into a human colon adenocarcinoma cell line results in marked increases in tumor growth rate and vascularization [61]. Furthermore, a recent report in this journal found levels of HE in malignant human breast tissues to be four times the levels in normal tissues from the same breast [62] and HE levels correlated with a marker for oxidative stress. HE-induced NO production by non-genomic pathways may contribute to breast cancer by initiating DNA damage, promoting growth and survival of initiated cells [44], and progression of cancer by increasing vascularization [61].

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