Hexachlorobenzene Is a Tumor Co-carcinogen and Induces Alterations in Insulin-Growth Factors Signaling Pathway in the Rat Mammary Gland

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Hexachlorobenzene (HCB) is a widespread environmental pollutant. Controversy still exists about the breast carcinogenic properties of organochlorines in humans. The ligands, receptors, and related signaling proteins of the insulin growth factor family are involved in the regulation of breast-cancer cell growth. The aims of this study were to determine: (1) whether HCB is co-carcinogenic in a medium term assay of N-nitrosoglutathione (NMU)-induced mammary tumors in rats; (2) the effect of HCB on insulin receptor (IR), insulin-like growth factor-I receptor (IGF-IR) and insulin receptor substrate-1 (IRS-1) levels and on IRS-1 phosphorylation; (3) microsomal and cytosolic Protein Tyrosine Kinase (PTK) activities in mammary glands and NMU-induced tumors. Sprague Dawley rats were injected with 50 mg/kg body weight of NMU at 50, 80, and 110 days old. HCB (100 mg/kg body weight) was administered three times a week from 65 to 110 days of age. Rats were separated in four groups: control, NMU, HCB, and NMU-HCB. HCB alone did not induce tumor development. Parameters of tumor development were increased in NMU-HCB compared to NMU rats. A higher cellular undifferentiation was observed in NMU-HCB tumors. IR, IGF-IR, and IRS-1 levels were higher in HCB than in controls. Conversely IGF-IR levels decreased in NMU-HCB vs. NMU group. The IRS-1 phosphorylation increased in HCB rats; however, it decreased in NMU-HCB vs. NMU. HCB decreased microsomal PTK activity in tumors. This study showed for the first time that HCB is a co-carcinogenic agent in NMU-induced mammary tumors in rats. Our results suggest that the IR and/or IGF-IR signaling pathway may be involved in the mechanism of action of HCB.

Key Words: hexachlorobenzene; N-nitroso N-methylurea; insulin receptor; insulin-like growth factor-I receptor; insulin receptor substrate-1; mammary tumors.

Breast cancer is an important cause of female morbidity. Many studies examined the possible association between organochlorinate pesticides exposure and content in adipose tissue of women suffering malignant and benign breast disease (Guttes et al., 1998), although the positive association between environmental exposure to these compounds and the risk of breast cancer is not yet clear (Zheng et al., 1999).

Hexachlorobenzene (HCB) is one of the most widespread environmental pollutants. Chronic administration of HCB to experimental animals elicits a number of effects, including hepatic porphyria (Mylchreest and Charbonneau, 1997), hypothyroxinemia (Kleiman de Pisarev et al., 1989; Rozman et al., 1986), and liver and thyroid carcinogenesis (Gustafson et al., 2000). Previous investigations have also shown that HCB administration to rats with spontaneous oestral cycles, resulted in significantly decreased uterine estrogen receptor levels and reduced circulating estradiol and pro lactin (Alvarez et al., 2000). HCB is a “dioxin-type” chemical and a weak agonist of the aryl hydrocarbon receptor (Ahr) (Hahn et al., 1989), a ligand-activated transcription factor. It has been proposed that the binding of “dioxin-type” chemicals to the Ahr complex could activate the phosphorylation of important proteins in the cytosol, plasma membrane, and other intracellular organelles, eliciting changes in signal transduction and gene expression activities (Matsumura, 1994). Previous works from our laboratory have shown that in vivo exposure to HCB caused profound changes in some biochemical parameters of rat liver membranes such as microsomal protein phosphorylation, downregulation of Epidermal Growth Factor Receptors (EGFR) and a dose-dependent increase in EGFR-tyrosine kinase activity (Randi et al., 1998, 2003). Insulin, insulin-growth factors (IGFs), and their corresponding receptors (IR, IGF-IR, and IGF-IIIR) participate in the growth and proliferation of normal and neoplastic mammary cells (Yee, 1994). The IR is a potential oncogene for mammary epithelial cells since its content is increased in most human breast cancer biopsies, and both ligand-dependent malignant transformation and ligand-dependent...
enhanced growth occurs in cultured breast cells overexpressing the IR (Frittitta et al., 1997). Insulin receptor substrate-1 (IRS-1) was originally identified downstream of the IR but can also be phosphorylated in response to IGF-IR activation (Sun et al., 1991). Following activation, IRS-1 binds a diverse set of downstream signaling molecules including p85, Grb2, Nck/Crk, Syk/Fyn, and SHP2 (Yenush and White, 1997). The complexity of IRS-1 upstream and downstream signaling reflects an emerging concept of complex cross-talk between extracellular and intracellular signaling pathways and may place IRS molecules in a central position to coordinate multiple signaling pathways (Pawson and Scott, 1997).

Experimental mammary tumor induction in rats by N-nitroso-N-methylurea (NMU) has been previously characterized. Proliferation and development of these experimental tumors may be regulated by complex interactions among several steroid and polypeptide hormones, as well as by growth factors (Martin et al., 1996, 1997). In the present study we assessed the possible co-carcinogenic action of HCB in a medium-term bioassay in NMU-induced mammary tumors in rats. Because IR, IGF-IR, and IRS-1 play an important role in both cell proliferation and cell transformation, we asked the question whether these biological parameters could be related to the mechanism of action of HCB in our experimental model.

MATERIALS AND METHODS

Reagents. Hexachlorobenzene was obtained from Máximo Paz S.A. (Argentina) with a purity of 99%. [γ-32P]ATP (6000 Ci/mmol) was purchased from NEN (Boston, MA). Mouse IgG monoclonal anti proliferative cell nuclear antigen (PCNA) was from Dako Laboratories (U.S.). The second antibody was purchased from Biotenx Laboratories (U.S.). Diaminobenzidine tetrahydrochloride (DAB) tablets were purchased from Sigma Chemical Co. (St Louis, MO). Anti-IRα goat polyclonal antibody (Santa Cruz. IRα 200 μg/ml). Rabbit polyclonal IGF-IR β (Cell Signaling Technology). Rabbit polyclonal anti-IRS-1 (Cell Signaling Technology). Monoclonal anti-phosphotyrosine PY20 (BD Transduction Laboratories). All reagents used were of analytical grade.

Animals. Virgin female Sprague-Dawley rats were randomly separated into batches of 4 rats, and housed in stainless steel cages with water and food ad libitum, at a temperature of 22–23°C, humidity of around 56% and 12-h light/dark cycle. All the procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council, USA.

Tumor induction. Mammary tumors were induced by ip injections of NMU as previously reported (Martin et al., 1996, 1997). Rats were randomly separated in four groups of 4 rats each, as indicated in the corresponding legends: (1) control rats; (2) NMU; (3) HCB; (4) NMU-HCB. NMU and NMU-HCB groups were ip injected with three doses of NMU (50 mg/kg body weight) at 50, 80, and 110 days of age as previously described. HCB (100 mg/kg body weight) was administered three times a week by gavage from 65 to 110 days of age, in accordance with an experimental design for the initiation-promotion studies reported by other authors (Gustafson et al., 2000). The total dose administered over the 45 days period was 1800 mg/kg body weight. The general health of the animals was not affected by the doses of HCB employed, as evaluated by the behavior and appearance of the rats: examination of the coat, mucous membranes, body weights, and food and water consumption. The low order of toxicity is indicative of the minimal absorption of HCB along the intestinal tract when administered in water. At doses ranging from 120–970 mg/kg body weight, only 2–5% of the administered HCB was absorbed from an aqueous suspension (Koss and Koransky, 1975).

The fungicide was prepared as a suspension of 4 mg/ml in water, containing Tween 20 (0.5 ml/100 ml). In previous studies we have also shown that HCB (100 mg/kg body weight) produced a down-regulation of the EGFR and a dose-dependent increase in EGFR-tyrosine kinase activity in the microsomal fraction of rat liver (Randi et al., 2003). Control animals received equal volumes of the appropriate solvent by the same route. The experimental design is represented in Figure 1. The development of breast tumors was examined by palpation, three times a week up to 150 days of age, after the first NMU injection. The size of tumors was measured with a calliper.

Tumor development. To evaluate mammary tumor development, the following parameters were determined: latency period (LP), as the number of days between the first NMU injection and the appearance of the first tumor in each rat; tumor incidence (TI), as the percentage of rats that developed at least one tumor; tumor number per rat (ntr), as the average number of tumors developed per rat; total tumor number, as the total number of tumors developed per batch; tumor volume was calculated as 4/3πr³; where r resulted from the average of half of the longest and the shortest tumor diameters. Tumor pattern was established in accordance with classification of rat mammary tumors defined by Russo and Russo (2000).

Histopathological studies. After an overnight fast, animals were sacrificed at 150 days of age. Mammary tissues from all the animals were removed for microscopic examination. Specimens for histopathological studies were fixed in 10% buffered formaldehyde and rehydrated with decreasing concentrations of alcohol from 100% to distilled water (10 min each). Then, slides were incubated at room temperature with normal goat serum for 30 min and incubated at 4°C overnight with anti-PCNA diluted with PBS and 4% BSA in a sealed humid chamber. Following incubation with the primary antibody the slides were washed with PBS three times for 10 min, incubated with the secondary biotinylated antibody in a humid chamber for 60 min (dilution 1:60), rinsed in PBS three times for 10 min and covered with streptavidine-peroxidase for 1 h at room temperature (dilution 1:20). Signal detection was carried out using a diamine benzidine reactive (DAB) substrate kit. Finally, the slides were washed and dried at 60°C and coverslipped using non-aqueous mountant (Akwik, synthetic balsame).

Cyclin-proliferating cell nuclear antigen (PCNA) immunohistochemical studies. Samples from mammary glands and mammary tumors were fixed for 6 h in 3.7% formaldehyde in 0.01 M phosphate saline buffer (PBS), pH 7.4, followed by immersion in ethanol 97% and embedded in paraffin. Serial sections (3–5 μm) were cut with a microtome; slides were deparaffinized in xylene (30–40°C) and rehydrated with decreasing concentrations of alcohol from 100% to distilled water (10 min each). Then, slides were incubated at room temperature with normal goat serum for 30 min and incubated at 4°C overnight with anti-PCNA diluted with PBS and 4% BSA in a sealed humid chamber. Following incubation with the primary antibody the slides were washed with PBS three times for 10 min, incubated with the secondary biotinylated antibody in a humid chamber for 60 min (dilution 1:60), rinsed in PBS three times for 10 min and covered with streptavidine-peroxidase for 1 h at room temperature (dilution 1:20). Signal detection was carried out using a diamine benzidine reactive (DAB) substrate kit. Finally, the slides were washed and dried at 60°C and coverslipped using non-aqueous mountant (Akwik, synthetic balsame).

FIG. 1. Experimental design. The initiating agent, NMU, was administered ip (50 mg/kg body weight) at 50, 80, and 110 days of age. HCB (100 mg/kg body weight) was delivered by gavage three times a week starting at 65 days of age. At 150 days the animals were sacrificed. The total dose administered over the 45 day period was 1800 mg/kg body weight. NMU ip; bold lines signify time period of vehicle administration; hatched lines signify time period of HCB administration.
Subcellular fractionation. Subcellular fractionation was performed using established procedures. Mammary tumors and normal glands were homogenized in 0.25 M sucrose, 0.05 M Hepes-Tris (pH 7.4) supplemented with the following protease inhibitors: 10 μg/ml bacitracin, 2 μg/ml apronitin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μM phenylmethylsulfonyl fluoride (PMSF) and 2 mM sodium orthovanadate, in an Elvehjem-type glass homogenizer (Randi et al., 2003). The crude homogenate was centrifuged for 20 min at 12,000 × g to remove the unbroken cells, nuclear fraction, and mitochondria. The resulting supernatant fluid was then centrifuged for 60 min at 100,000 × g to obtain the microsomal and cytosolic fraction.

Protein tyrosine kinase assay. Protein tyrosine kinase activity in breast tissue microsomes and cytosol was assayed with the synthetic peptide poly-[Glu80 Na, Tyr20] (poly GT), as described by Hilf et al. (1988), with some modifications. Briefly, the microsomes were resuspended in 125 mM Hepes, 0.25% Triton (pH 7.4) with protease inhibitors and sodium orthovanadate as described before. Microsomal and cytosolic proteins were diluted in a solution of ionic buffer (10 mM MnCl2, 50 mM MgCl2, 10 mM p-nitro phenyl phosphate (PNPP), and 5% glycerol). Phosphorylation of poly-GT, was carried out in a reaction mixture containing 10 μg of microsomal or 20 μg of cytosolic proteins for tumor samples and 10 μg of microsomal or 40 μg of cytosolic proteins for mammary glands samples, 250 μg of poly-GT, 10 μCi of [γ-32P]ATP (40 μM). Incubation times were 10 min for mammary gland and 5 min for tumors at 28°C in a final volume of 50 μl. The protein phosphorylation reaction was stopped by the addition of 20 μl of a solution containing 20 μM EDTA, 200 mM NaF, 40 mM Na2PO4, 40 mM NaPO4, 40 mM Hep, and 0.4% Triton. Thirty μl of the mixture was then placed on a Whatman 3MM paper and washed four times with a solution containing 10% TCA and 1 mM Na2PO4. The filters were dried for 1 h at 80°C. The radioactivity associated to the filter was measured by liquid scintillation counting in water. Assays in which poly-GT was omitted were used as blanks. Phosphorylation assays were linear up to 5–10 min.

Polyacrylamide gel electrophoresis. Microsomal proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli (1970), with a 6% resolving gel and a 4% stacking gel, in Mini-Protean II electrophoresis cell (Bio-Rad). The 0.75 mm slab gels were electrophoresed 45–60 min at a constant voltage setting of 150 V, at 4°C. The running buffer (5X) used was 0.12 M Tris base, 1 M glycine, and 10% SDS (pH 8.3). The molecular weight of the different bands of proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein phosphorylation reaction products were visualized by autoradiography after being transferred to a nitrocellulose membrane. The PVDF membrane was incubated with rabbit polyclonal anti-IGF-IR (1:500) (Cell Signaling Technology). The immunoblot was then washed seven times with TBST (TBS with 0.1% Tween 20) at 37°C, and the bound antigen-antibody complexes were detected by incubation for 1 h with antibody to rabbit immunoglobulin G (1:1000) (ECL, Amersham Biosciences, Inc., horseradish peroxidase linked, whole donkey antibody NA934V). The membrane was stripped and reprobed with rabbit polyclonal anti-IRS-1 (1:500) (Cell Signaling Technology).

To determine IRS-1 phosphorylation, the membrane was stripped and reprobed with mouse monoclonal antiphosphotyrosine PY20 (1:1000) (BD Transduction Laboratories, 1 mg/ml) for 2 h. The immune complexes were visualized employing the enzyme-linked enhanced chemiluminescence kit (ECL, Amersham Biosciences, Inc.) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.

RESULTS

Parameters of Tumor Development

Mammary gland carcinogenic potential of HCB was assessed by comparing the latency period, tumor incidence, tumor number per rat, and tumor volume. As shown in Figure 2A, HCB increased tumor incidence significantly in NMu-HCB group as compared to NMu group. At the end of 100 days post-first NMU injection, all the animals of the NMu-HCB group had developed tumors while only 62% had developed tumors in the NMu group at that time. During the observation period NMu-HCB rats showed a significantly greater number of tumors per rat than NMu rats (Fig. 2B). Figure 2C showed that tumor volume was significantly higher in NMu-HCB group than in NMu group. At the end of the observation period, the tumors from NMu-HCB rats were 5-fold bigger than tumors of NMu rats. The latency period was not altered in the NMu-HCB group compared to NMu group (81.8 ± 3.3 vs. 82.1 ± 2.5 days respectively, NMu, n = 8; NMu-HCB, n = 6). Rats receiving only HCB did not develop mammary tumors.

Histological Observations

Histological observations showed evident differences in the growth pattern of tumors of NMu and NMu-HCB groups. All tumors were mammary ductal carcinomas with different degree of cellular atypia and with cribriform, papillary, or comedocarcinoma pattern. Figures 3A and 3B illustrate characteristic adenocarcinomas developed in mammary gland of NMu rats, both with atypical mitotic cells. Tumors from NMu rats showed a predominant proportion of cribriform pattern as we have
previously reported (Martin et al., 1996, 1997). Conversely, tumors developed in NMU-HCB rats showed preponderantly an undifferentiated pattern, with higher cellular atypia, number of mitosis, anisocariosis and higher proportion of solid tumor areas (Figs. 3C and 3D). One important finding was the increased proportion of papillary and comedocarcinoma tumor type in NMU-HCB rats versus NMU (Table 1). In all cases the stromal connective tissue was mainly composed by mast cells, lymphocytes, eosinophils, and polymorphonuclear leukocytes.

Normal mammary glands showed a single epithelial layer and scarce myoepithelial cells surrounding the ductal lumens (Fig. 3E). A significant increase in thickness of stromal walls of epithelial mammary ducts as well as in the number of ducts were observed in the mammary glands of HCB treated rats (Fig. 3F).

**PCNA Immunohistochemical Studies**

Cyclins are a family of proteins implicated in the induction and control of mitosis, activating cell division cycle (cdc) kinases. PCNA which accumulates progressively through interphase is commonly used as a cellular proliferation marker (Busmanis, 1998). PCNA positive epithelial cells for 200 epithelial cells in mammary tumors indicated the presence of a higher number of positive epithelial cells in NMU-HCB tumors versus NMU. (NMU: 119 ± 5; NMU-HCB: 140 ± 11*). (*Significantly different from NMU, *p* < 0.05, *t*-test.)

**Protein Tyrosine Kinase Activity**

PTK activities in mammary glands and tumors have been investigated using poly-GT as an exogenous substrate. Our results showed that HCB did not alter microsomal PTK activity compared to the control group; however, HCB decreased microsomal PTK activity (0.6-fold) in NMU tumors (Fig. 4A). NMU treatment increased cytosolic PTK activity compared to mammary gland (10-fold). However HCB did not change cytosolic PTK activity compared to the control or NMU group (Fig. 4B).

**Insulin Receptor Content in Normal Mammary Glands and Tumors**

At first, to evaluate the effect of HCB on insulin signaling pathway, we investigated whether the IR was affected by HCB treatment in mammary gland and in NMU-induced tumors. IRs were immunoprecipitated from the microsomal fraction of glands and tumors and then immunoblotted with anti-αIR

![FIG. 2. Tumor development parameters. Rats of NMU and NMU-HCB groups were ip injected with three doses of NMU (50 mg/kg body weight) at 50, 80, and 110 days of age. HCB (100 mg/kg body weight) was administered three times a week by gavage from 65 to 110 days of age. All animals were examined by palpation three times a week up to 100 days post-first NMU injection to detect mammary tumors. (A) Tumor incidence vs. time (days post-first NMU injection). Statistical comparisons were made by analysis of variance (two-way ANOVA) with a 95% confidence interval. ANOVA: interaction, *p* = 0.0385; treatment factor: *p* = 0.0469; time factor: *p* = 0.0001. Nonparametric post-hoc test was performed to identify significant differences for treatment, between mean values of NMU-HCB vs. their corresponding time-matched controls (*p* < 0.02). (B) Tumor number per rat versus time (days post-first NMU injection). Statistical comparisons were made by analysis of variance (two-way ANOVA) with a 95% confidence interval. ANOVA: treatment factor: *p* = 0.002; time factor: *p* = 0.0001. Bonferroni post-hoc test was performed to identify significant differences for treatment, between mean values of NMU-HCB vs. their corresponding time-matched controls (*p* = 0.05). (C) Tumoral volume (cm³) versus time (days post-tumor appearance). Statistical comparisons were made by analysis of variance (two-way ANOVA) with a 95% confidence. ANOVA: interaction, treatment factor and time factor *p* < 0.0001. ANOVA analysis was followed by *t*-test for treatment comparisons (**p** < 0.0001). The values are mean ± SEM of two experiments with *n* = 4 rats per group.
specific antibody. As shown in Figures 5A and 5B, the IR content was significantly increased (1.4-fold) in HCB and (0.6-fold) in NMU vs. control rats. No significant changes were observed in IR content of NMU-HCB tumors compared to NMU.

**Insulin-like Growth Factor-I Receptors Levels in Normal Mammary Glands and Tumors**

Because IGF-IR plays an important role in the development of tumor growth in breast cancer, we decided to study IGF-IR content in normal glands and tumors. Microsomal fractions from mammary glands and tumors were electrophoresed and immunoblotted with anti-IGF-IR antibody. As shown in Figures 6A and 6B, IGF-IR content was significantly increased in mammary glands from HCB treated rats (2.4-fold) and in NMU tumors (4.7-fold) when compared to control mammary gland. In tumors, the response to HCB was different, because HCB induced a remarkable decrease of IGF-IR content (8-fold).
HCB Effects on the Insulin Substrate Receptor–1 Content and Its Phosphorylation Status

Due to the fact that alteration of IRS-1 levels can affect the sensitivity and response to both insulin and IGF-I, we examined the ability of HCB to alter IRS-1 content and its phosphorylation in mammary glands and tumors. We demonstrated that IRS-1 protein levels were increased in HCB mammary gland compared to control (3-fold). A nonsignificant increase in IRS-1 content was observed in NMU-HCB vs. NMU group. However while not significant, the direction of the response was consistent with what was observed in the mammary gland (Figs. 7A and 7B).

IRS-1 phosphorylation was calculated by dividing the arbitrary units of the scanned autoradiograph of phosphorylated IRS-1 by the amount of IRS-1 present on the immunoblot probed with IRS-1 antibody. Our results showed that IRS-1 tyrosine phosphorylation was increased by HCB (0.5-fold) in mammary gland. Conversely IRS-1 tyrosine phosphorylation was reduced by HCB in NMU tumors (10-fold) (Figs. 8A and 8B).

**DISCUSSION**

Many epidemiological studies have examined the correlation between HCB levels in serum samples or breast tissue with cancer risk (Charlier et al., 2003; Guttes et al., 1998; Zheng et al., 1999). Those studies suggested that HCB may occur in higher concentrations in samples from breast cancer patients than controls. However, other authors do not support a positive association between environmental exposure to HCB and risk.

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cribiform</th>
<th>Papillary</th>
<th>Comedocarcinoma</th>
</tr>
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<tbody>
<tr>
<td>NMU</td>
<td>17/20 (85%)</td>
<td>2/20 (10%)</td>
<td>1/20 (5%)</td>
</tr>
<tr>
<td>NMU-HCB</td>
<td>27/44 (61%)</td>
<td>13/44 (30%)**</td>
<td>4/44 (9%)**</td>
</tr>
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*Note. Proportion of tumors of each type developed in NMU-HCB and NMU rats.

**Indicated significantly different from NMU (p < 0.01) by Fisher test.

**FIG. 4.** Effects of NMU and HCB on PTK activity of mammary glands and tumors. Mammary glands and tumors were homogenized and microsomal and cytosolic fractions were obtained. PTK activity was determined using poly GluTyr as substrate. The data are expressed as the mean ± SEM of two independent experiments of 4 rats per group. (A) Microsomal PTK activity. *Significantly different from NMU (p < 0.05); (B) Cytosolic PTK activity. **Significantly different from control (p < 0.001) and ***Significantly different from HCB (p < 0.001). Statistical comparisons were made by analysis of variance (one-way ANOVA) with a 95% confidence interval followed by Tukey post-hoc test to identify significant differences between mean values and indicated controls.

**FIG. 5.** IR levels in mammary gland and tumors. The IR was immunoprecipitated from microsomes of mammary glands and tumors by a specific antibody. (A) Immunohistochemical detection of IR levels. The IR immunocomplex was resolved on a 6% SDS-PAGE, and IR was detected by western blotting using a specific antibody. A goat anti-rabbit IgG antibody conjugated with peroxidase was used to detect the primary antibody binding. (B) Quantification of microsomal IR levels by densitometry scanning of the immunoblots of three independent experiments. Values are means ± SEM. **Significantly different from control (p < 0.001). Statistical comparisons were made by analysis of variance (one-way ANOVA) with a 95% confidence interval followed by Tukey post-hoc test to identify significant differences between mean values and indicated controls.

IRS-1 protein levels were increased in HCB mammary gland compared to control (3-fold). A nonsignificant increase in IRS-1 content was observed in NMU-HCB vs. NMU group. However while not significant, the direction of the response was consistent with what was observed in the mammary gland (Figs. 7A and 7B).

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of breast cancer (Zheng et al., 1999). Considering the existent controversy about the breast carcinogenic properties of organochlorinated compounds in humans, new investigations are necessary to clarify this point.

Hormone dependence and receptor proteins in NMU-induced mammary tumors have been previously studied in our laboratory (Martin et al., 1998). These tumors express estrogen receptors (ER), progesterone receptors (PgR), and EGFR. ER content was found to be independent of tumor histopathological variety (Martin et al., 1996).

In the present study we investigated the interaction of HCB and NMU on the mammary gland carcinogenic process in a medium-term bioassay in rats. The results of our study showed that the number of tumors per rat, tumoral incidence, and tumoral volume were increased by HCB in NMU-induced mammary tumors. A substantial reduction in the tumor incidence and tumor number/rat was observed in NMU-HCB vs. NMU group, at 70 days. At the present time we do not have an explanation for that striking effect.

Histopathological observations in mammary gland indicated that HCB induced an increase in the number and thickness of ducts, and a higher cellular atypia in tumors from NMU-HCB rats when compared to NMU group. These observations together showed for the first time that HCB has a tumor co-carcinogenic activity. Further studies will be necessary to determine HCB levels in rat serum and mammary glands and tumors, in order to extrapolate our experimental data to effects of HCB in women with regard to breast cancer.

Although not specific to breast cancer, the role of HCB in tumor promotion model systems have been reported in a combined experimental and simulation approach of hepatic preneoplastic foci development (Ou et al., 2003).

Insulin exerts a significant mitogenic action in normal mammary tissue and breast cancer cells in culture (Belfiore et al., 1996) and has been shown to potentiate mitogenic influence of other growth factors (Finlayson et al., 2003). Frittitta et al. (1993) demonstrated that in breast cancer there is an increased number of structurally and functionally normal IRs. In some breast cancer tissues, however, the sensitivity of the receptor tyrosine kinase activity to insulin was greatly increased (Frittitta et al., 1993). In the present study, we found that the IR content was significantly increased in mammary glands from HCB and tumors from NMU treated rats. However IR content was not significantly altered by HCB administration in NMU tumors. Liu and Safe (1996) found that 2,3,7,8-dibenzod-p-dioxin (TCDD), the most toxic congener of the “dioxin type” chemicals, induced IR mRNA levels in MCF-7 cells. Further studies are needed to clarify if those motifs are involved in HCB mechanism of action.
Protein tyrosine kinases play a major role in the transduction of intracellular mitogenic signals, and are also involved in the process of cellular transformation. It has been reported that NMU-induced mammary carcinoma which expresses both particulate and cytosolic forms of PTKs, may be acutely regulated in vivo and could play an important role in mammary carcinogenesis (Srivastava et al., 1991). In the present study we have shown that HCB decreased microsomal PTK activity in NMU-induced tumors, without changes in cytosolic PTK activity. Other authors reported that human malignant breast cancer exhibited considerably higher tyrosine kinase activities in both cytosol and membrane fractions, compared to benign breast tumors (Hennipman et al., 1989). The changes observed in PTK activity may be attributable to the presence of several tyrosine kinase-receptors, such as EGFR and/or insulin growth factor receptors.

On this respect we have previously reported that HCB induced downregulation of EGFR and increased EGFR-tyrosine kinase activity in rat liver, as well as time and dose dependent changes in membrane protein phosphorylation, suggesting that protein kinase and phosphatase activities could be involved in the pleiotropic effect of HCB (Randi et al., 1998, 2003). It has been shown that EGFR were expressed in NMU-induced mammary tumors in rats (Martin et al., 1998).

The role of IGFs in the process of tumor growth has received much attention with the finding that raised circulating IGF-I concentrations correlate very closely with the relative risk for the development of several common cancers including cancers of the breast (Yee, 1994). In this work we showed an increased content of IGF-IR in mammary gland of HCB treated rats. However HCB decreased IGF-IR content in NMU-HCB induced tumors. On this respect a strong down-regulation of IGF-IR was observed in advanced human breast cancer (Schnarr et al., 2000).

The view emerging from cellular and animal studies is that abnormally high levels of IGF-IRs may contribute to the increase of tumor mass and/or aid tumor recurrence, by promoting cell survival, and cell-cell interactions. However, IGF-IR expression for diagnosis and prognosis of breast cancer is still a matter of debate because of conflicting results. In breast cancer, except for the well-established correlations with estrogen receptor status, the association of the IGF-IR with other prognostic parameters are still insufficiently documented (Surmacz et al., 1998).

IGF-1 is the predominant signaling molecule activated by IGF-I, insulin and interleukin-4 in ER-positive human breast cancer cells (Jackson et al., 1998). IRS-1 initiates a signaling cascade that includes the MAP kinase and PI3 kinase pathways, that ultimately results in cell proliferation. Understanding the regulation of IRS-1 production and tyrosine phosphorylation will contribute toward elucidating the mechanisms that control IGF signaling in the mammary gland and tumors. With this in view, we investigated the ability of HCB to modulate IRS-1 protein levels and tyrosine phosphorylation.

In this study we demonstrated that the increase in IGF-IR content is accompanied by an increase in IRS-1 phosphorylation, in mammary gland from HCB treated rats. Conversely a decrease in IGF-IR content in NMU-HCB tumors is correlated with a decrease in IRS-1 phosphorylation. A strong down-regulation of IGF-IR and IRS-1 in advanced human breast tumors has been reported by Schnarr et al. (2000). It has been shown that constitutive activation of IRS-1 is a frequent event in human tumors, but it was found that the modulation in the degree of IRS-1 phosphorylation, does not necessarily correlate with total IRS-1 phosphorylation (Chang et al., 2002).

Several mechanisms appear to be involved in inhibiting IGF-stimulated tyrosine phosphorylation of IRS-1, including phosphatase-mediated dephosphorylation (Noguchi et al., 1994),
and serine/threonine phosphorylation mediated by kinases like MAPK (De Fea and Roth, 1997). The decrease in IRS-1 phosphorylation may also involve altered expression of secreted IGFBPs by HCB. Further studies will be necessary to determine which signalling pathway(s) may be implicated in IRS-1 decreased phosphorylation in NMU-HCB tumors.

Together, these studies demonstrated for the first time that HCB enhanced the development and malignancy of NMU-induced mammary tumors in rats. Furthermore our results showed that HCB altered insulin/IGF-I signaling pathway, at least with regard to IR and IGFR-I expression as well as IRS-1 content and tyrosine phosphorylation. These observations suggest that the IGF-IR signaling pathway may be involved in HCB tumor co-carcinogenic action.

SUPPLEMENTARY DATA

Supplementary data are available online at www.toxsci.oxfordjournals.org.

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REFERENCES


