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Alterations in c-Src/HER1 and estrogen receptor α signaling pathways in mammary gland and tumors of hexachlorobenzene-treated rats

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ABSTRACT

Hexachlorobenzene (HCB) is an organochlorine pesticide that acts as an endocrine disruptor in humans and rodents. The development of breast cancer strongly depends on endocrine conditions modulated by environmental factors. We have demonstrated that HCB is a tumor co-carcinogen in rats and an inducer of proliferation in MCF-7 cells, in an estrogen receptor α (ERα)-dependent manner, and of migration in MDA-MB-231 breast cancer cell line. In the present study, we examined HCB effect on c-Src/human epidermal growth factor receptor (HER1) and ERα signaling pathways in mammary glands and in *N*-nitroso-*N*-methylurea (NMU)-induced mammary tumors in rats. Furthermore, we evaluated histopathological changes and serum hormone levels. Rats were separated into four groups: control, HCB (100 mg/kg b.w.), NMU (50 mg/kg b.w.) and NMU-HCB. Our data show that HCB increases c-Src and HER1 activation, c-Src/HER1 association, and Y699-STAT5b and ERK1/2 phosphorylation in mammary glands. HCB also enhances Y537-ERα phosphorylation and ERα/c-Src physical interaction. In tumors, HCB also induces c-Src and HER1 activation, c-Src/HER1 association, as well as T308-Akt and Y699-STAT5b phosphorylation. In addition, the pesticide increases ERα protein content and decreases p-Y537-ERα levels and ERα/c-Src association in tumors. HCB increases serum 17-beta estradiol and prolactin contents and decreases progesterone, FSH and LH levels in rats without tumors, while the opposite effect was observed in rats with tumors. Taken together, our results indicate that HCB induces an estrogenic effect in mammary gland, increasing c-Src/HER1 and ERα signaling pathways. HCB stimulates c-Src/HER1 pathway, but decreases ERα activity in tumors, appearing to shift them towards a higher malignancy phenotype.

1. 1-Introduction

Persistent organic pollutants such as polychlorinated biphenyls, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and hexachlorobenzene (HCB) are ubiquitous chemical compounds that bioaccumulate and persist in the environment. Organochlorine compounds have numerous actions, including antiestrogenic or estrogenic effects that disrupt reproductive function (Soto et al., 1998).

HCB is a dioxin-like compound and a weak ligand of the aryl hydrocarbon receptor (AhR) protein (Hahn et al., 1989). Its presence in the environment is due mainly to its industrial and agricultural applications. Chronic exposure of laboratory animals to HCB elicits a number of effects such as thyroid dysfunctions (Chiappini et al., 2009), porphyria (Mylchreest and Charbonneau, 1997) and thyroid adenomas (Courtney, 1979), and it is also a promoter of liver foci growth (Ou et al., 2001) and rat mammary tumors (Randi et al., 2006). The International Agency for Research on Cancer classifies HCB as a probable human carcinogen (ATSDR, 2002).

Exposures to endocrine-disrupting compounds are suspected of contributing to increased breast cancer incidence as well as precocious puberty (Fenton et al., 2005). Rat mammary carcinogenesis is one of the most widely used surrogate models, because it closely mimics the human disease allowing elucidation of the influence of host factors. Tumors can be chemically induced by the administration of either dimethylbenz[*a*]anthracene or *N*-nitroso-*N*-methylurea (NMU; Russo and Russo, 2000). Terminal end buds (TEBs) highly proliferative structures present in the mammary

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gland in the peripubertal period, disappear from the mature gland when differentiation proceeds. Several studies have determined that TEBs structures are sensitive to chemical carcinogen in rodent models, and their presence at the time of carcinogen exposure is positively associated with tumor multiplicity (Russo and Russo, 1996).

Breast cancer is a sex-dependent malignancy influenced by a myriad of hormones and growth factors. Estrogens, which are necessary for the normal development of both reproductive and non-reproductive organs, including the mammary glands, mainly exert their physiological effects by binding to their specific receptors, estrogen receptor α (ERα) or β (ERβ), though they may act as well through alternate non-receptor mediated pathways. Membrane-associated ERα transduces estrogen signals rapidly, leading to the activation of many signaling molecules, such as MAPK, Akt, $p21^{ras}$, Raf-1 and protein kinase C and the release of nitric oxide and stimulation of prolactin (Prl) secretion (Cheskis, 2004). Human epidermal growth factor receptor (HER1) is also activated under 17-beta estradiol (E_2) rapid action, leading to activation of its downstream MAPK and Akt signaling pathways (Kahlert et al., 2000).

c-Src kinase is overexpressed or highly activated in carcinomas of the breast, lung, colon, skin, cervix, and gastric tissues (Biscardi et al., 2000). c-Src is directly associated with many growth factor receptors and signaling molecules, and has a central role in E_2 cytoplasmic signaling. E_2-stimulated proliferation and activation of intracellular pathways such as MAPK and signal transducers and activators of transcription (STATs) are severely blocked in the presence of c-Src inhibitors or kinase-dead c-Src. Because c-Src activity is restricted by intramolecular interactions, association of ER/c-Src and scaffold proteins may serve to stimulate c-Src activity and activate intracellular signaling pathways, including PI3K and MAPK (Fox et al., 2009). ERα is phosphorylated in Y537 by c-Src *in vivo*, and this phosphorylation is required for triggering DNA synthesis and tumor growth (Varricchio et al., 2007). In a recent study, we found that HCB induces c-Src activation, Y537-ERα phosphorylation and cell proliferation in MCF-7 human breast cancer cell line (García et al., 2010).

HER1 stimulates tumor growth and progression by activating several signaling pathways associated with cell proliferation, angiogenesis, invasion and metastasis (Sebastian et al., 2006). Several non-physiologic agents such as radiation, oxidants and alkylating compounds induce ligand-independent activation of HER1 (Knebel et al., 1996). Y845-HER1 phosphorylation induced by c-Src is proposed to facilitate cross-talk between HER1 and signaling pathways activated by other cellular receptors, such as G-protein-coupled receptors and ERα. c-Src cooperates with HER1 in growth signaling, which might be particularly relevant in breast cancer in which both proteins are frequently upregulated (Biscardi et al., 2000). In a previous study, we found that HCB enhances both c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways and cell migration in MDA-MB-231 human breast cancer cell line (Pontillo et al., 2011).

The aim of the present investigation was to study HCB-mechanism of action on c-Src/HER1 and ERα signaling pathways, analyzing ERK1/2, Akt and STAT5b downstream signaling in mammary glands and in NMU-induced mammary tumors in rats. We also sought to link these pathways with histopathological parameters and serum hormone levels.

2. Materials and methods

2.1. Reagents

Hexachlorobenzene (>99% purity, commercial grade) and NMU were obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Anti-HER1, anti-c-Src, anti-phospho-Y416-c-Src, anti-Akt2, anti-phospho-T308-Akt, anti-phospho-T202/Y204 ERK 1/2 and anti-histone 2B antibodies were purchased from Cell Signaling Technology Inc. (MA, USA). Anti-ERK 1/2, anti-STAT5b and anti-phospho-Y699-STAT5b were obtained from Upstate (Lake Placid, NY, USA), and anti-phospho-Y845-HER1 and anti-phospho-Y537-ERα antibodies were purchased from Abcam Ltd (Cambridge, UK). Anti-β-actin was obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-ERα monoclonal antibody was purchased from Chemicon International Inc. (Temecula, CA, USA). The A/G plus agarose protein was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies goat anti-Mouse and goat anti-Rabbit IgG (H+L)-HRP conjugate and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad Laboratories (CA, USA). The enhanced chemiluminescence kit (ECL) and molecular weights standards Full-range Rainbow were purchased from GE Healthcare Life Sciences (Buckinghamshire, UK). All other chemicals used were of analytical grade.

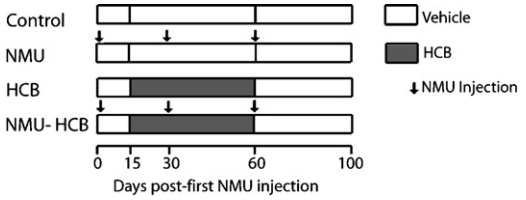

Fig. 1. Experimental design. NMU (50 mg/kg body weight) was administered i.p. at 50, 80 and 110 days of age. HCB (100 mg/kg body weight) was delivered by gavage three times a week starting at 65 days of age. At approximately 150 days, when the animals were found to be in oestrus phase, they were sacrificed. Total HCB dose administered over the 45 day period was 1800 mg/kg body weight. NMU i.p.; bold lines signify time period of vehicle administration; hatched lines signify time period of HCB administration.

2.2. Animals

Virgin female Sprague–Dawley rats were randomly separated into batches of 6 rats, and housed in stainless steel cages with water and food *ad libitum*, at a temperature of 22–23 °C, 45–75% humidity 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.

2.3. Tumor induction

Mammary tumors were induced by intraperitoneal (i.p.) injections of NMU as previously reported (Martin et al., 1997). Rats were randomly separated in four groups of 6 rats, as indicated in the corresponding legends: (1) control; (2) NMU; (3) HCB; (4) NMU-HCB. NMU and NMU-HCB groups were i.p. 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, in accordance with our previous study, in which we have shown that HCB at the same dose increases the number of tumors and tumor volume in chemically induced-rats (Randi et al., 2006). 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 dose 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 to 970 mg/kg body weight, only 2–5% of the administered HCB is absorbed from an aqueous suspension (Koss and Koransky, 1975). The agent was prepared as a suspension of 4 mg/ml in water, containing Tween 20 (0.5 ml/100 ml). Control animals received equal volumes of the appropriate solvent by the same route. The experimental design is represented in Fig. 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, also measuring the size of tumors with a calliper.

2.4. 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; number of tumors per rat (n/r), as the average number of tumors developed per rat; total tumor number, as the total number of tumors developed per batch; tumor volume, calculated as $4/3\pi r^3$, where r resulted from the average of half of the longest and the shortest tumor diameters.

2.5. Histopathological studies

After an overnight fast, animals were sacrificed and mammary tissues from all the animals were extracted for microscopic examination. Specimens for histopathological studies were fixed in 10% buffered formaldehyde (pH 7.4), embedded in paraffin, sectioned and stained with hematoxylin–eosin (HE) or toluidine blue (TB),