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Chronic exposure to hexachlorobenzene results in down-regulation of connexin43 in the breast

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ABSTRACT

Decreased expression of connexins has been associated with cancer, but the underlying mechanisms are poorly understood. We have previously shown that a 5 day exposure to hexachlorobenzene (HCB) resulted in decreased connexins expression in hepatocytes 45 days later, and that this down-regulation was linked to activation of Akt through the ILK pathway. Because HCB promotes cancer in both the liver and breast, the present study aimed to determine if the mechanisms are similar in both tissues. MCF-12A breast cells were thus transfected with vectors coding for either Akt or a constitutively active form of Akt. In those cells, activation of Akt was correlated with decreased Cx43 levels. Female rats were then exposed to HCB by gavage either following the same protocol used previously for the liver or through a chronic exposure. While no changes were observed after the 5 days exposure protocol, chronic exposure to HCB resulted in increased Akt levels and decreased Cx43 levels in breast cells. *In vitro*, Akt was activated in MCF-12A cells exposed to HCB either for 7 days or chronically, but no changes were observed in junctional proteins. Together, these results suggested that, while activation of Akt can decrease Cx43 expression in breast cells *in vitro*, other mechanisms are involved during HCB exposure, leading to a decrease in Cx43 levels in a model- and duration-dependent manner. Finally, we showed that HCB effects are tissue specific, as we did not observe the same results in breast and liver tissues.

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1. Introduction

Hexachlorobenzene (HCB) is a widespread environmental pollutant that has been used as a fungicide and is a by-product of many industrial processes. Although its use has been banned in many countries, it bioaccumulates mainly due to its high lipid solubility and resistance to degradation (Tong and Yuan, 2012). As a result, it is still found in human tissues, including the breast, as well as in breast milk (Luzardo et al., 2013; Hernandez et al., 2009). Studies in rodents have demonstrated that HCB is an epigenetic carcinogen that promotes tumorigenesis in both the liver and breast (Randi et al., 2006; Gustafson et al., 2000; Cabral et al., 1996). The effects of HCB in the breast have been studied primarily by the Kleiman de Pisarev and Randi group, who examined mechanisms of HCB carcinogenicity in rats and in two breast cancer cells lines, MDA-MB-231 and MCF-7 cells (Randi et al., 2006; Garcia et al., 2010; Pontillo et al., 2011, 2013; Pena et al., 2012). They showed that HCB can increase cell proliferation and activate, among others, the estrogen receptor (ER), the insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor (Garcia et al., 2010); as well as the epidermal growth factor receptor (EGFR) and the aryl hydrocarbon receptor (AhR) (Pontillo et al., 2011). However, the mechanisms linked to HCB carcinogenicity are still poorly understood.

Gap junctions are transmembrane channels formed by a family of proteins called connexins (Cxs). In humans and rodents, there are 20 and 21 members, respectively, each of which are encoded by different genes and have specific tissue distributions (Laird, 2006). Gap junctions allow for the exchange of small molecules, such as metabolites, secondary messengers and electrical signals, between adjacent cells, a process known as Gap Junctional Intercellular Communication (GJIC). This intercellular communication is crucial for many tissue functions, as well as for cellular proliferation, differentiation and apoptosis (Herve and Derangeon, 2013). Not surprisingly, mutations in Cxs have been associated with human diseases, including cancer (Su and Lau, 2014). While Cx26 and Cx43, two Cxs that are expressed in breast tissue, were proposed as tumor suppressors in the early 1990s, their role in breast cancer remains poorly understood (Lee et al., 1992., 1991; Naus and Laird, 2010). Previous studies have shown that Cx26 and Cx43 are down-regulated or mis-localized in breast tumors from human patients (Lee et al., 1992, 1991; Laird et al., 1999). In tumor cell lines, re-expression of Cxs results in a re-differentiation toward a more

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normal phenotype (McLachlan et al., 2007; Hirschi et al., 1996), whereas normal cell lines become more migratory and invasive upon Cxs down-regulation (Shao et al., 2005). Our recent data have shown that decreased Cx43 levels in mammary glands results in hyperplasia and an increased number of metastases in the lung in a breast cancer animal model (Plante et al., 2011), confirming the potential link between Cx43 and cancer in the breast.

Similarly, decreased Cxs expression or GJIC has been suggested to be a mechanism linked to chemically induced carcinogenesis years ago (Paul, 1986; Trosko et al., 1994; Yamasaki, 1995). Since then, many studies have demonstrated that exposure to various chemical compounds can promote cancer by modulating gap junction function or Cxs regulation in various tissues (Chipman et al., 2003). We previously conducted a series of experiments to determine the mechanisms involved in HCB-induced hepatocarcinogenesis. Using both *in vivo* and *in vitro* models, we showed that exposure to HCB resulted in decreased hepatic Cxs (Cx26 and Cx32) expression and inhibition of GJIC in treated rats 45 days after the last HCB dose (Plante et al., 2002, 2005). There was also a concomitant decrease in expression of E-cadherin and a nuclear translocation of β-catenin, two components of adherens junctions. Many studies have suggested that components of adherens and gap junctions are regulated by common but unknown mechanisms and that their loss is linked to tumor progression (Foroni et al., 2012; Miyoshi and Takai, 2005; Giepmans, 2004). We demonstrated that in HCB-exposed animals, the inhibition of Cx32 and E-cadherin resulted from the activation of the Integrin-linked-kinase (ILK) pathway, but through two different effectors, Akt and GSK3β, respectively (Plante et al., 2005, 2006, 2007). Akt is activated in multistep manner that initially involves ATP-dependent phosphorylation. Once activated, Akt goes, in a first step, to the membrane where it is phosphorylated by different proteins, including ILK; it then translocates into the nucleus, where it participates in gene regulation (Saji et al., 2005; Andjelkovic et al., 1997). Thus, phosphorylated Akt (p-Akt) is thought to play a crucial role in cell proliferation and differentiation.

Importantly, although HCB is a tumor promoter, the original protocol used to induce hepatocarcinogenesis (named “ABC”) was not a typical tumor “initiation–promotion–progression”, as animals were exposed to HCB 95 days prior to tumor initiation by diethynitrosamine (DEN) (Larouche, 1993; Krishnan et al., 1991). Thus, those findings suggested that HCB caused structural changes to the liver prior to the tumor initiation that render the liver more susceptible to tumor development.

Because HCB promotes both liver and breast cancer, and because down-regulation of Cx32 and Cx43 is associated with cancer in the liver and breast, respectively, the present study aimed to determine if HCB can act through similar mechanisms in both tissues. More specifically, we wanted to determine whether exposure to HCB render breast cells more susceptible to tumorigenesis by activating ILK pathway, similar to what was observed 45 days after the exposure in liver. We thus used *in vivo* and *in vitro* models to assess the effects of HCB on ILK, GSKβ, Akt, Cx43, E-cadherin and β-catenin levels in mammary glands, as well as in a non-tumorigenic human breast cell line.

2. Materials and methods

2.1. Animals treatments

Female Sprague-Dawley rats (180–200 g) were purchased from Charles River Canada (St Constant, Quebec, Canada). Rats were maintained under a constant photoperiod of 12 h light:12 h dark and received food and water ad libitum. Rats were administered HCB (100 mg/kg) or vehicle (corn oil, controls) by gavage for 5 consecutive days and sacrificed 45 days after the last dose of HCB, as previously described (protocol ABC) (Plante et al., 2002, 2005). Alternatively, rats were administered HCB (100 mg/kg) or vehicle (corn oil, controls) by gavage twice a week for 50 days (protocol HCB). At the time of sampling, rats were anesthetized by isoflurane inhalation and bled. The mammary glands were removed and processed for epithelial cells isolation. All animal protocols used in this study were approved by the University Animal Care Committee.

2.2. Isolation of epithelial cells from mammary glands and protein extraction

Epithelial cells were isolated as described with slight modification (McGrath et al., 1985). Briefly, lymph nodes were removed from freshly isolated mammary glands. Mammary gland tissues were then minced and digested in 25 ml of collagenase solution (200 U/ml collagenase 1A; 8 U/ml hyaluronidase IV; 8 KU/ml DNAse I; 6.7% fetal bovine serum (FBS) in DMEM/F12 media) for two hours at 32 °C at 200 rpm. 25 ml of 2X isolation buffer were added (0.02 mM EGTA; 10 μg/ml Insulin; 2 U/ml heparin; 5% FBS in DMEM/F12 media). The cell suspension was then filtered through a 74 μm nylon mesh before being centrifuged for 10 min at 800 rpm. The pellet was resuspended in 1 ml of 1X isolation buffer containing 1 mg/ml DNAse 1 and 4% $MgCl_2$ and incubated for 15 min at room temperature. The cell suspension was layered on a Percoll gradient (4 ml Percoll and 6 ml isolation buffer centrifuged at 20,000 rpm for 30 min) and centrifuged at 4 °C at 3000 rpm for 20 min. The fraction containing epithelial cells was collected, washed with PBS, and resuspended in lysis buffer (25 mM HEPES; 150 mM NaCl; 10 mM $MgCl_2$; 1 mM EDTA; 0.1% Triton 100-X; 10% glycerol; 10 μg/ml aprotinin; 1 mM sodium vanadate). After 10 min of incubation at 4 °C, the cells were sonicated and centrifuged for 10 min at 4 °C at 3000 rpm. The supernatant, which contained epithelial cell proteins, was aliquoted and stored at −80 °C.

2.3. Cell culture

MCF-12A cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium nutrient mixture (DMEM/F12) without phenol red, supplemented with 5% normal horse serum, 20 ng/ml epidermal growth factor, 500 ng/ml hydrocortisone, 10 μg/ml insulin and 100 ng/ml cholera toxin. All cells were grown in 10 cm diameter plastic tissue culture dishes kept in a humidified incubator with 5% CO_2 at 37 °C. MCF-12A cell lines were plated at 0.56 million cells per dish.

2.3.1. Short term HCB treatment

Treatments were initiated on subconfluent cell cultures (24 h after plating) by the addition of media containing 0.1% (v/v) dimethyl sulfoxide (DMSO) as a vehicle, 30 μM or 50 μM HCB in DMSO, as previously described (Plante et al., 2005). Cells were treated for 7 days and the media was changed twice. 5 replicates of the DMSO- and HCB-treated groups were independently processed.

2.3.2. Long term HCB treatment

Treatments were initiated on subconfluent cell cultures (24 h after plating) by the addition of media containing 0.1% (v/v) DMSO or 20 μM HCB in DMSO. Cell cultures were thereafter continuously exposed to DMSO or HCB and maintained for 50 days in culture with passages every 2 or 3 days. 3 replicates of DMSO- and HCB-treated groups were grown independently. Cell counts and viability were assessed using trypan blue dye exclusion and a hemocytometer. After 50 days in culture, treatments were stopped and

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