Hexachlorobenzene-Induced Early Changes in Ornithine Decarboxylase and Protein Tyrosine Kinase Activities, Polyamines and c-Myc, c-Fos and c-Jun Proto-Oncogenes in Rat Liver

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Hexachlorobenzene (HCB) is a lipophilic chemical compound that is widely distributed in the environment. HCB is known to cause liver tumors in experimental animals. In the present study the in vivo effect of HCB treatment on ornithine decarboxylase (ODC) and protein tyrosine kinase (PTK) activities, free polyamine content, and c-Myc, c-Fos, and c-Jun protein levels in rat liver were investigated. HCB (1000 mg/kg body weight) increased hepatic immunodetectable c-Myc, c-Fos, and c-Jun levels after 6 h, and ODC activity and spermine and putrescine content after 18 and 24 h, while maximum stimulation of PTK activity occurred at 12 h. PTK and ODC activities varied in a dose-dependent manner. The time-course of c-Myc, c-Fos, and c-Jun protein levels was different for each proto-oncogene. They were all elevated at the second day of treatment, while only c-Fos and c-Jun remained elevated after 10 days of HCB exposure. These data jointly suggest that the increase in ODC activity may be the consequence of proto-oncogene induction. The alterations in PTK activity suggest that the growth factor signal transduction pathway may be involved in the regulation of the proto-oncogene levels or/and ODC activity. The decrease in PTK activity after the first day, even in the presence of α-D-Difluoromethylornithine (DFMO), an inhibitor of ODC activity, suggests that it is not regulated by polyamines. These results may be relevant to the early molecular events involved in HCB tumor promoter activity in rat liver.

Key Words: hexachlorobenzene; rat liver; ODC activity; PTK activity; polyamine content; proto-oncogene levels.

Hexachlorobenzene (HCB) is one of the most widespread environmental pollutants. Although the use of HCB was discontinued in most countries in the 1970s, it is still released into the environment as a byproduct of the manufacture of other polychlorinated organo-compounds (Courtney, 1979). Chronic exposure of laboratory animals to HCB elicits a number of harmful effects such as thyroid dysfunctions (Kleiman de Pisarev et al., 1990), porphyria (Sopena de Krakoff et al., 1994), and thyroid adenomas (Courtney, 1979). Human exposure to HCB has been associated with soft-tissue sarcoma, breast tumors (Iscan et al., 2002), and brain and thyroid cancer (Grimalt et al., 1994). Hexachlorobenzene is also known to cause liver-cell tumors in animals (Cabral and Shubik, 1986; Cabral, et al., 1996). It has been proved that HCB is a promoter of diethylnitrosamine (DEN)-initiated hepatocarcinogenesis (Stewart et al., 1989). HCB is a “dioxin-type” chemical and a weak agonist of the aryl hydrocarbon receptor (Ahr) (Hahn et al., 1994), a ligand-activated transcription factor. It has also 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 pathways and gene expression (Matsumura, 1994).

Most tumor promoters induce ornithine decarboxylase (ODC) in vivo (Kitchin and Brown, 1989). ODC catalyzes the formation of putrescine and CO₂ from ornithine and is the first and key regulatory enzyme in polyamine biosynthesis. ODC and polyamines are known to play a pivotal role in cell proliferation and to contribute to the development of cancer (Heby and Persson, 1990; Pegg, 1988). Celano et al. (1989) suggested that individual polyamines may play a selective role in the expression of specific growth-related genes. In normal cells, the activity of ODC shows a rapid, transient increase upon stimulation by growth factors (Heby and Persson, 1990; Pegg, 1988), whereas the cells transformed by chemical carcinogens and oncogenes such as v-src, neu, and ras seem to have a constitutively elevated ODC activity (Gilmour et al., 1986; Hölttä et al., 1994). Recently, it has been postulated that the ODC gene may act as an oncogene, because the overexpression of this gene is essential for cell transformation (Shanz and Pegg, 1994).

ODC activity has been shown to have multiple levels of
control ranging from transcriptional regulation (Katz and Kahana, 1987) to posttranslational modification (Ray et al., 1999). A relevant signal associated with cell growth, differentiation, and malignant transformation is the induction of a group of immediate-early (IE) genes that encode the transcriptional activator proteins c-Fos, c-Jun, and c-Myc (Schütte et al., 1989; Vogt and Bos, 1989). Interaction of c-Fos with any one of the various members of the Jun family forms the activating protein-1 (AP-1) transcription factor required for cell proliferation (Abate et al., 1990).

Transcription regulatory factors encoded by early-response genes might control the expression of delayed-early response genes, such as ODC (Desiderio et al., 1998). Tyrosine-kinase receptors trigger intracellular signals by coupling to multiple signal transduction pathways. Mitogenic activated protein kinases (MAPK) phosphorylate and regulate protein kinases involved in the control of gene expression (Whitmarsh and Davis, 1996). Ornithine decarboxylase activity can be regulated by phosphorylation, confirming the pivotal role of ODC in cell-signaling pathways triggered at the cell surface (Desiderio et al., 1998).

As HCB is a nongenotoxic tumor promoter, it is reasonable to ask whether it can initiate the signals associated with cell growth and transformation. We have previously demonstrated that HCB induces time- and dose-dependent alterations, both in rat hepatic microsomal protein phosphorylation and protein tyrosine kinase (PTK) activity, during the early stages of HCB administration (Randi et al., 1998). We have also reported that HCB changes epidermal growth factor (EGF) affinity for its membrane receptor, induces EGFR internalization and stimulates EGFR-tyrosine kinase activity (Randi et al., 1998, 2003). Other subcellular mechanistic studies have shown that HCB exposure induced alterations in intercellular gap junctional communication in female rat liver (Mally and Chipman, 2002; Plante et al., 2002).

The aim of this study was to investigate if alterations of ODC activity and proto-oncogenes proteins (c-Fos, c-Jun, and c-Myc) could be involved in the early stages of HCB exposure. Our results showed, for the first time, that HCB in vivo treatment elicited very early changes in the induction of a group of immediate-early genes and ODC activity that could be relevant in the early molecular events involved in HCB tumor promoter activity in rat liver.

MATERIALS AND METHODS

Chemicals. Hexachlorobenzene (> 99% purity, commercial grade) was a gift from Máximo Paz S.A. (Buenos Aires, Argentina). L-1-14C-Ox ornithine (56 Ci/mol) and γ-32P-ATP (6000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). c-Myc (monoclonal) antibody was a gift from Dr. Rosa Bergoc, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina; c-Fos and c-Jun (polyclonal) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). α-Difluoromethylornithine (DFMO) was a gift from Merrell Dow Research Institute (Cincinnati, OH). All other chemicals were ACS grade from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Animals and treatment. Female Wistar rats (160–180 g at the onset of the experiment) were purchased from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. The rats were fed Purina 3 rat chow (Cabeça S.C.A, Argentina) and water ad libitum. Environmental conditions consisted of a 12-h light-dark cycle, 20–24°C, and 45–75% humidity. Following a 7-day acclimation period, HCB (1000 mg/kg body weight) and other doses as indicated in the text, were administered daily through a stomach tube. HCB (40 mg/ml) was suspended in water containing Tween 20 (0.5 ml/100 ml). Control animals received equal volumes of appropriate solvent by the same route. For time-course studies, the animals received HCB (1000 mg/kg body weight) for 1, 2, and 10 days. The acute exposure assay was carried out at 6, 12, 18, and 24 h posttreatment. This dose of HCB was chosen based on our recent results that showed that the maximum increase in rat liver EGFR-tyrosine kinase activity was reached at this concentration (Randi et al., 2003). For the dose-response assay, HCB was administered for 24 h at 10, 100, and 1000 mg/kg body weight. ODC and PTK activities and free polyamine content were evaluated in the same group of six animals.

To inhibit ODC activity in vivo, we used DFMO, an irreversible specific suicide inhibitor and analogue of ornithine (Prakash et al., 1980). This soluble product was administered to rats, in water beverage at a concentration of 2%, 24 h before starting the treatment with HCB (1000 mg/kg body weight). Following an overnight fast, the animals were killed by decapitation, and the livers perfused through the portal vein with 0.154 M NaCl.

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, including examination of their coats, mucous membranes, and body weights, and their food and water consumption.

Hepatic microsomal fraction preparation. Liver microsomal membranes were isolated from a crude homogenate as described by Randi et al. (2003).

Protein tyrosine kinase assay. PTK activity assay in rat liver membranes was based on that described by Tremblay and Beliveau (1984). Phosphorylation of the synthetic peptide, poly-[Glu80 Na, Tyr20] (poly GT), was carried out in a reaction mixture containing 80 μg/100 μl of particulate proteins, 500 μg/100 μl of poly GT, 8 μM of γ-32P-ATP (40 μM), 10 mM MnCl2, 20 mM MgCl2, 0.2 mM Na3VO4, and 25 mM HEPES-Tris (pH 7.0).

Preparation of nuclear protein extracts. Nuclear proteins were isolated from rat liver according to the procedure of Gorski et al. (1986) with modifications. Briefly, the tissue was homogenized in a Dounce-type glass homogenizer with a Teflon pestle in three volumes of homogenization buffer (0.25 M sucrose, 10 mM NaCl, 5 mM MgCl2, 50 mM Tris, pH 7.5, and 0.2% Triton) and filtered through moistened gauze. The filtrate was centrifuged for 4 min at 1,225 × g and the pellet was resuspended in 2 volumes of homogenization buffer without Triton and centrifuged at 1,225 × g for 4 min. The resulting pellet was resuspended in the same buffer and centrifuged at 100,000 × g for 60 min on a 2 M sucrose layer in homogenization buffer. Pure nuclei were treated with lysis buffer (0.3 M KCl, 1 mM MgCl2, 20 mM Tris–HCl, 2 mM DTT, pH 8.0) for 40 min and centrifuged at 1,075 × g for 15 min. The supernatant containing the nuclear protein extract was stored at −80°C. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Western blot analysis. Nuclear protein extracts were electrophoresed in a 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane (PVDF, Bio-Rad). Filters were blocked with 1% fat-free dried milk, 1% BSA in TBST (10 mM Tris, 150 mM NaCl and 0.05% Tween 20, pH 8.0). Blocked membranes were washed five times with TBST for 10 min and incubated with the first antibody (1:400) in TBST overnight. The membranes were washed five times with TBST and incubated with the suitable horseradish peroxidase-conjugated anti-species-specific antibody. Immunoblots were then washed five times with TBST and once with TBS. Proteins were visualized employing the enzyme-linked enhanced chemiluminescence kit (ECL, Amersham Biosciences, Inc., UK) and quantified by scanning laser densitometry in a Fotodyne (Foto/Analyst), Gel-Pro Analyzer 3.1.
Polyamine determination. Putrescine, spermidine, and spermine were extracted with 0.2 N perchloric acid at 4°C. Supernatants were obtained after centrifugation at 1,075 g for 15 min and treated with dansyl-chloride according to Seiler’s method (Seiler, 1970).

Determination of ODC activity. ODC activity was determined by measuring 14C-CO2 release from L-1-14C-ornithine. Liver was homogenized in a cold extraction buffer containing 50 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol (DTT), 1 mM pyridoxal phosphate, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 2 mM EDTA, 2 mM DTT, 50 μM pyridoxal phosphate, 1 mM PMSF, 1 mM ornithine, and 0.2 μCi L-1-14C-ornithine, and the enzyme preparation (0.4 – 0.8 mg) in a final volume of 0.8 ml. The incubation was carried out at 37°C for 1 h and stopped by adding 30% trichloracetic acid (TCA) and incubated for an additional 30 min. 14CO2 released was trapped on hyamine-soaked Whatman 1 filter paper and counted for radioactivity in a scintillation counter. Blanks did not contain enzyme preparation. The enzyme activity was expressed in terms of nmols/mg protein/h.

Statistical analysis. Data from dose and time-course effects were analyzed by one-way ANOVA, followed by Tukey’s post-hoc test. Other statistical analyses were performed using Student’s t-test, as indicated in the text. Differences between control and treated animals were considered significant when p values were < 0.05.

RESULTS

Effects of HCB on ODC and PTK Activities and Free Polyamine Content in Rat Liver

To evaluate time-dependent effects of HCB on PTK and ODC activities, the rats received HCB (1000 mg/kg body weight), administered daily through a stomach tube for 1, 2, and 10 days. Data represent the means ± SEM of six animals. PTK activity was measured using poly Glu-Tyr as exogenous substrate, while ODC activity was assayed using L-1-14C-ornithine, as indicated in Materials and Methods. Significantly different from control values at: a = p < 0.05 and b = p < 0.0005 (one-way ANOVA). Tukey post-hoc test was used to compare with control only.

HCB Increases c-Myc, c-Fos and c-Jun Protein Levels

Considering that the transforming activity of ODC may be mediated by the enhanced expression of c-Myc and/or other growth-related genes, we determined c-Myc, c-Fos, and c-Jun protein levels in liver obtained from control and HCB-treated rats (1000 mg/kg body weight), at 1, 2, and 10 days of exposure.

As shown in Figure 3, the immunodetectable proto-oncogene levels showed time-dependent changes, with a maximum increase on the second day. It is noteworthy that c-Jun showed a remarkable increase (5-fold) at this time, when compared with c-Fos and c-Myc. The protein levels of c-Fos/c-Jun re-
mained significantly elevated at 10 days. c-Myc exhibited a transient increase at 2 days and returned to basal values later.

**HCB Effects on ODC and PTK Activity, and Polyamine Content Are Dose Dependent**

Rats were intoxicated with HCB (10, 100, or 1000 mg/kg body weight) or vehicle and were sacrificed after the first day of treatment, when ODC activity was at its highest value.

ODC activity increased in a dose-dependent manner. This increase was significant at doses of at least 100 mg/kg body weight; a maximum effect was observed at HCB 1000 mg/kg body weight. Conversely, liver microsomal PTK activity decreased significantly at the same concentrations (Fig. 4).

Regardless of the dose, the spermidine content remained constant. However, as previously described, spermine and putrescine contents were increased significantly by HCB 1000 mg/kg body weight. At lower doses (100 mg/kg body weight HCB) only putrescine was increased (Fig. 5).

**ODC and PTK Activities in DFMO-Treated Rats**

The aim of this experiment was to determine whether the reduction elicited by HCB in the PTK activity correlated with

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**FIG. 3.** Time-course changes on hepatic c-Myc, c-Jun, and c-Fos protein levels in HCB-treated animals. The animals were treated daily with HCB (1000 mg/kg body weight) or vehicle by gavage and sacrificed at 1, 2, and 10 days. The hepatic nuclear proteins were extracted and analyzed on a 10% SDS-polyacrylamide gel, and the levels of c-Myc, c-Jun, and c-Fos were detected by western blotting using specific antibodies. The secondary antibody conjugated with peroxidase was used to detect the primary antibody binding, as described in Materials and Methods. (A) Immunoblotting of c-Myc, c-Jun, and c-Fos levels. (B) Densitometric quantification of proto-oncogene content by scanning of the immunoblots. Values are means ± SEM of three rats. Values significantly different from control data are indicated by a = p < 0.05; b = p < 0.01 and c = p < 0.001. Tukey post-hoc test was used to compare with control only.

**FIG. 4.** HCB-induced dose-dependent alterations in hepatic ODC and PTK activities after 24 h of treatment. The corresponding HCB dose (10, 100, or 1000 mg/kg body weight) or vehicle was administered by gavage, and the animals were sacrificed at 24 h. PTK activity was measured using poly Glu-Tyr as exogenous substrate, while ODC activity was assayed using L-1-14C-ornithine, as indicated in Materials and Methods. Data represent the means ± SEM of six animals. Significantly different from control values at: a = p < 0.05 and b = p < 0.0005 (one-way ANOVA). Tukey post-hoc test was used to compare with control only.
the increase in polyamine levels due to ODC activation. We used DFMO as a specific inhibitor of ODC activity. DFMO does not interfere with other enzymatic reactions in which ornithine is the substrate (Jänne et al., 1991). The ability of DFMO to inhibit both ODC and growth in various mammalian cells and organs has been reported before (Prakash et al., 1980).

The following experimental groups were used: Control (rats received water containing Tween 20 by gavage 24 h before sacrifice); DFMO (rats received 2% DFMO in drinking water until sacrifice, 48 h later; 24 h before sacrifice they received water containing Tween 20 by gavage); HCB (rats received 1000 mg/kg body weight of HCB by gavage and were sacrificed 24 h later); and HCB + DFMO (rats received 2% DFMO in drinking water until sacrifice 48 h later; 24 h before sacrifice they were administered HCB at a dose of 1000 mg/kg body weight by gavage).

Our results showed that PTK activity was reduced significantly both in Control and HCB-treated rats, when ODC activity was knocked out by DFMO, suggesting that there is no direct link between these two enzymes (Fig. 6).

**HCB Acute Exposure Induced Changes in Hepatic c-Myc, c-Jun and c-Fos Protein Levels**

We analyzed the time-course of the response of the immunodetectable c-Myc, c-Jun, and c-Fos levels at very early stages of HCB (1000 mg/kg body weight) exposure (6, 12, 18, and 24 h posttreatment). Figure 7 shows that the kinetics of the response to HCB was markedly different for the three proto-oncogenes studied. HCB significantly increased c-Myc, c-Jun, and c-Fos levels at 6 h after exposure. c-Jun decreased significantly thereafter to levels below control at 24 h, while c-Myc returned to control levels after 18 h. Conversely, c-Fos levels increased steadily in a time-dependent manner after 6 h.

**Time-Course of ODC and PTK Activities and Free Polyamine Content at Very Early Stages of HCB Exposure**

Figures 8 and 9 show the alterations in ODC and PTK activities and free polyamine content in an acute exposure assay (6, 12, 18, and 24 h posttreatment with HCB). A remarkable 12-fold increase in ODC activity was detected after 18 h, remaining significantly higher than control after 24 h of treatment. On the other hand, PTK activity was significantly increased at 12 h posttreatment, decreasing thereafter.

Putrescine levels rose 6-fold after 18 h, and this increase continued after 24 h, as shown in Figure 9, although with lower values. Putrescine levels strongly correlated with ODC activity. Spermine content was significantly increased in a time-dependent manner after 18 h. The spermidine levels did not change significantly.

**DISCUSSION**

Our time-course studies showed that the main effect of HCB on hepatic ODC activity was elicited at early stages of exposure (18 h). ODC-induced activity was evidenced in a concomitant increase in putrescine levels. The increase in spermine levels could be the result of HCB stimulation of S-adenosyl-
methionine decarboxylase (SAMDC) activity, since ODC and SAMDC are commonly regulated by the same external stimuli (Heby and Persson, 1990). Naturally occurring polyamines, putrescine, spermidine, and spermine, are known to accumulate in cancer cells (Tabib and Bachrach, 1999) and could be involved in enhancing the transcription or translation of proto-oncogenes. Celano et al. (1989) suggested that individual polyamines may have a selective role in the expression of specific growth-related genes. Whether the elevated levels of putrescine and spermine at 18 and 24 h are causally related to the increase in c-Jun at 2 days remains to be determined.

The early significant increase in hepatic immunodetectable proto-oncogene levels (6 h) followed by the induction of ODC activity at 18 h, suggests that the enhancement in ODC activity may be the consequence of the induction of one or several proto-oncogenes. Another possibility is that upregulation of c-Fos/c-Jun and ODC follow two independent pathways, as proposed by Jansen et al. (1999). ODC activity may be only induced by c-Myc, in agreement with Arora et al. (2000), who reported that c-Myc is an important positive regulator of cell

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![FIG. 7. HCB acute exposure-induced enhancement in hepatic c-Myc, c-Jun, and c-Fos levels. The animals were treated with HCB (1000 mg/kg body weight) or vehicle by gavage and were then sacrificed at 6, 12, 18, and 24 h. The hepatic nuclear proteins were extracted and analyzed on a 10% SDS-polyacrylamide gel, and the levels of c-Myc, c-Jun, and c-Fos were detected by western blotting using specific antibodies. The secondary antibody conjugated with peroxidase was used to detect the primary antibody binding, as described in Materials and Methods. (A) Immunohistochemical detection of c-Myc, c-Jun, and c-Fos levels (B) Densitometric quantification of proto-oncogene content by scanning of the immunoblots. Values are means ± SEM of three rats. Values are expressed as fold changes over untreated rats. Values significantly different from control data are indicated by $p < 0.05$; $b = p < 0.01$ and $c = p < 0.001$ (one-way ANOVA). Tukey post-hoc test was used to compare with control only.]

![FIG. 8. HCB acute exposition-induced changes in hepatic ODC and PTK activities. The animals were treated with HCB (1000 mg/kg body weight) or vehicle by gavage and then were sacrificed at 6, 12, 18, and 24 h. PTK activity was measured using poly Glu-Tyr as exogenous substrate, while ODC activity was assayed using L-1-14C-ornithine, as indicated in Materials and Methods. Data represent the means ± SEM of six animals. Significantly different from control values at: $a = p < 0.01$ and $b = p < 0.001$ (one-way ANOVA).]
proliferation in rat liver. Bartolome et al. (1999) showed that insulin-stimulated ODC mRNA expression and enzymatic activity in the rat liver are accompanied by a concomitant increase in the expression of c-Myc and Max mRNAs, which are known to act physiologically as transcriptional activators of the ODC gene (Bello-Fernández et al., 1993). Pereira et al. (2001) reported that tumor promoters increased mRNA expression of c-Myc in liver. Further studies are necessary to understand whether the observed increase in ODC activity found in this work is related to the enhancement in the proto-oncogene/s levels.

c-Myc increases transiently at very early times (6, 12 h), while c-Jun is remarkably increased at 2 days. Conversely c-Fos increases steadily from the beginning of HCB exposure. These results suggest that HCB deregulates each proto-oncogene in a different manner. Future experiments are necessary to determine whether the elevated c-Fos and c-Jun levels encountered at 10 days are biologically relevant. It is interesting that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a well-known proven tumor promoter and teratogen, also induced ODC activity (Raunio and Perlkonen, 1983) and the expression of c-fos, c-jun, jun-B and jun-D proto-oncogenes at early exposure times (Puga et al., 1992).

We have previously shown that chronic treatment with HCB produced downregulation of EGFR and dose-dependent increases in EGFR-tyrosine kinase activity in rat liver (Randi et al., 2003). Our results showing an early increase in PTK activity (12 h), followed by a peak in ODC activity at 18 h, suggest that ODC could be regulated by phosphorylation. The growth factor signal transduction pathway may be involved in the activation of "primary response genes" through a series of phosphorylations and dephosphorylations of cytosolic and eventually nuclear proteins, as suggested by Matsumura (1994).

Our studies demonstrate, for the first time, that HCB stimulated early increases in proto-oncogene levels, ODC activity, and free polyamine content in rat liver. The induction of proto-oncogenes may be relevant to the early molecular events involved in HCB tumor promoter activity. Although our results are obviously far from establishing a direct connection between the induction of proto-oncogene and such an activity, they provide the groundwork for further promising research in this area.

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REFERENCES


