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Effects of γ-hexachlorocyclohexane and L-3,3',5-triiodothyronine on rat liver cytochrome P4502E1-dependent activity and content in relation to microsomal superoxide radical generation

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ABSTRACT

Liver microsomal cytochrome P4502E1-dependent p-nitrophenol (PNP) hydroxylation and expression of cytochrome P4502E1 were studied in rats subjected to γ-hexachlorocyclohexane (HCCH) or L-3,3',5-triiodothyronine (T_3) administration as a possible mechanism contributing to superoxide radical ($O_2^{\cdot-}$) generation. HCCH treatment (a single dose of 40 mg/kg body wt) produced a 43% increase in the content of total cytochrome P450, whereas T_3 (daily doses of 0.1 mg/kg body wt for two consecutive days) led to a 37% decrease. NADPH-dependent $O_2^{\cdot-}$ generation was elevated by HCCH and T_3, expressed as either per mg of protein or per nmol of cytochrome P450, with a 135% enhancement in the $O_2^{\cdot-}$ production/superoxide dismutase (SOD) activity ratios being observed in both conditions. This was partly due to depression of SOD activity. Concomitantly, the molecular activity of NADPH-cytochrome p450 reductase was enhanced by 90 and 69% by HCCH and T_3, respectively. In these conditions, microsomal PNP hydroxylation showed increases of 58 and 45% in HCCH- and T_3-treated rats over control values, respectively, with a parallel 31% (HCCH) and 41% (T_3) enhancement in the content of cytochrome P4502E1 assessed by western immunoblotting. We conclude that HCCH and T_3 enhance the expression and activity of cytochrome P4502E1 and that of NADPH-cytochrome P450 reductase in rat liver, regardless of the changes in total cytochrome P450 content, representing major contributory mechanisms to microsomal NADPH-dependent $O_2^{\cdot-}$ generation.

Key terms: γ-Hexachlorocyclohexane; L-3,3',5-Triiodothyronine; Superoxide radical; Cytochrome P4502E1; Rat liver

INTRODUCTION

Reactive species derived from chemicals, oxygen, or nitrogen have been implicated as putative noxious intermediates responsible for cellular damage (Comporti, 1989; Radi et al., 1991). Because electrophilic metabolites or radicals and excited species can readily interact with essential biomolecules, covalent binding to cellular components and/or their oxidative modification can occur, leading to structural and functional alterations when protective mechanisms are overcome or exhausted (Comporti, 1989; Kappus, 1987). Previous work by our group has shown that acute γ-hexachlorocyclohexane (HCCH or lindane) treatment enhances the oxidative stress status of the liver, an effect that is both dose- (Junqueira et al., 1986) and time-dependent (Junqueira et al., 1988; Barros et al., 1988) and that seems to be primarily related to its biotransformation by the microsomal P450 system. The reductive activity of this pathway generates electrophilic intermediates of HCCH (Baker

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et al., 1985) which are conjugated with glutathione (GSH) for elimination as mercapturates (Kurihara et al., 1979), thus inducing an early (4-6 h) phase of oxidative stress involving GSH depletion (Barros et al., 1988) with a lipid peroxidative response (Junqueira et al., 1988). Induction of cytochrome P450 at later times after HCCH intoxication (24 h) further enhances the oxidative stress status of the liver due to acceleration of xenobiotic biotransformation (Videla et al., 1990) and higher microsomal NADPH-dependent superoxide radical ($O_2^{\cdot-}$) generation (Junqueira et al., 1988), with higher oxidation index (Fernández et al., 1999). Furthermore, the time course of the changes in parameters related to oxidative stress induced by HCCH coincides with the onset and progression of morphological lesions in the liver, which return to normal depending on the hepatic content of the insecticide and on the extent of microsomal production of $O_2^{\cdot-}$ (Junqueira et al., 1988; 1997).

From the toxicological point of view, cytochrome P4502E1, the ethanol-inducible form (Lieber, 1997), is considered of particular interest due to (a) its capacity to oxidize several chemicals (i.e., ethanol, carbon tetrachloride, acetaminophen, nitrosamines) to reactive intermediates that are hepatotoxic (Guengerich et al., 1991; Koop, 1992; Lieber, 1997), and (b) its poor coupling with NADPH-cytochrome P450 reductase (Gorsky et al., 1984; Ekström and Ingelman-Sundberg, 1989). The latter feature of cytochrome P4502E1 involves a high NADPH oxidase activity, with enhanced production of $O_2^{\cdot-}$ and hydrogen peroxide (H_2O_2) during microsomal NADPH oxidation, and represents an effective catalyst for lipid peroxidation (Ekström and Ingelman-Sundberg, 1989; Dai et al., 1993). In view of these considerations, the aim of this study was to evaluate the influence of HCCH treatment on liver microsomal $O_2^{\cdot-}$ production in relation to cytochrome P4502E1-dependent activity and content of this specific isoenzyme. To achieve this aim, liver microsomal hydroxylation of p-nitrophenol (PNP)(Reinke and Moyer, 1985) and immunoblot analysis (Towbin et al., 1979) were performed in control and HCCH-treated rats, and results were compared to values found in animals after L-3,3',5-triiodothyronine (T_3) administration, which is known to induce oxidative stress in the liver involving enhanced microsomal $O_2^{\cdot-}$ generation (Fernández et al., 1985; Massa and Fernández., 1999).

MATERIALS AND METHODS

Animals and treatments

Male Sprague-Dawley rats weighing 200-250 g were given free access to food and water and maintained on a 12 h light/dark cycle. Animals received a single i.p. injection of 40 mg HCCH/kg body weight or equivalent volumes of corn oil (HCCH diluent, controls). A separate group of rats were given daily i.p. doses of 0.1 mg T_3/kg body weight for two consecutive days or equivalent volumes of 0.1 N NaOH (T_3 diluent, controls). Determinations were performed 24 h after HCCH or T_3 treatment, time at which the hepatic content of HCCH was assayed by a gas chromatography technique (Bainy et al., 1993), the levels of T_3 in serum were measured by radioimmunoassay (GammaCoat™ [^{125}I]T_3 Kit, Baxter Healthcare Corp., Cambridge, MA), and the thermogenic status of the animals was assessed by measurements of their rectal temperature (Fernández et al., 1985). All animals used received humane care according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health publication No. 86-23).

Biochemical parameters

Livers were homogenized in 150 mM KCl containing 5 mM Tris, pH 7.4. Microsomal fractions were prepared by ultracentrifugation and washed once before suspension in 120 mM potassium phosphate buffer, pH 7.2. These samples were used for the determination of the total content of cytochrome P450 (Omura and Sato, 1964), the production of $O_2^{\cdot-}$ by determining the

superoxide dismutase-sensitive rate of adrenochrome formation at 485 nm (Boveris et al., 1983), and the activity of NADPH-cytochrome P450 reductase by monitoring the reduction of cytochrome c at 550 nm (Williams and Kamin, 1962). Post-microsomal cytosolic fractions were used to determine superoxide dismutase (SOD) activity (Misra and Fridovich, 1969).

Oxidation of PNP is a suitable assay for the determination of the microsomal activity dependent on cytochrome P4502E1 (Reinke and Moyer, 1985; Lieber, 1997). This was assayed by measuring the A_{546} of the 4-nitrocathecol produced, and activity was calculated using the extinction coefficient of 10.28 $mM^{-1}cm^{-1}$ (Reinke and Moyer, 1985). For Western immunoblotting, the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Proteins separated were electrotransferred to a nitrocellulose membrane as described by Towbin et al. (1979), with the Trans-Blot Cell system (Bio-Rad, Hercules, CA) in transfer buffer. The membranes were incubated with primary anti-rat cytochrome P4502E1 antibody (dilution 1:500), using acetone treated rat liver microsomes containing cytochrome P4502E1 (0.1 µg/blot) as a standard (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan). This was followed by incubation with anti-rat IgG antibody coupled to horseradish peroxidase (dilution 1:5000) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and subsequent detection of luminol reactive bands with 4-chloro-1-naphtol and hydrogen peroxide. The protein content of microsomal and cytosolic fractions was determined according to Lowry et al. (1951).

Statistical analysis

Values shown correspond to the means ± SEM for the number of separate experiments indicated. The statistical significance of differences among multiple groups was carried out by one-way ANOVA followed by the Newman-Keuls' test, and is indicated by the letters identifying each experimental group.

RESULTS AND DISCUSSION

Administration of HCCH to rats resulted in a higher content of the insecticide in the liver compared with that in control animals [controls, not detectable (n=4); HCCH-treated rats, 5.81 ± 1.14 µg/g wet liver (n=5)]. In these conditions, HCCH led to a 43% increase ($p<0.05$) in the content of total cytochrome P450 of the liver (Fig. 1A), in agreement with earlier reports (Srinivasan and Radhakrishnamurty, 1983; Junqueira et al., 1988).

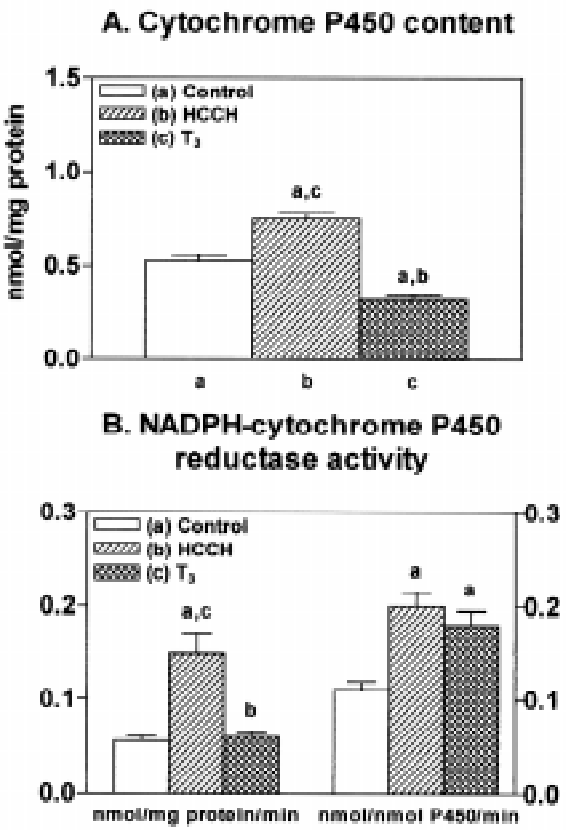

Figure 1. Content of cytochrome P450 (A) and NADPH-cytochrome P450 reductase activity (B) in liver microsomal fractions of control rats and animals subjected to γ-hexachlorocyclohexane (HCCH) or L-3,3',5-triiodothyronine (T_3) administration. Control rats for HCCH and T_3 treatments exhibited comparable values ($p>0.05$) and constitute a single group. Values shown correspond to means ± SE for 7-15 animals per experimental group. The significance of the differences between mean values ($P< 0.05$) was assessed by one-way ANOVA and the Newman-Keuls' test, and it is shown by the letters identifying each experimental group.

Concomitantly, the specific activity of hepatic NADPH-cytochrome P450 reductase is enhanced by 171% following HCCH administration, over control values, with a 90% increase being found when expressed in terms of cytochrome P450 content (molecular activity) (Fig. 1B). T_3 administration, involving higher levels of the hormone in serum [controls, 48 ± 5 ng/dl (n=5); T_3-treated rats, 277 ± 35 (n=5); $p<0.05$] and enhanced rectal temperature of the animals [controls, 36.9 ± 0.02 °C (n=5); T_3-treated rats, 38.2 ± 0.02 (n=12); $p<0.05$], elicited a 37% decrease ($p<0.05$) in the cytochrome P450 content of the liver (Fig. 1A). Although T_3 administration for two consecutive days failed to modify the specific activity of NADPH-cytochrome P450 reductase (Fig. 1B), which is enhanced after T_3 treatment for three consecutive days (Simon-Giavarotti et al., 1998), the molecular activity of the enzyme is elevated by 69% (Fig. 1B). HCCH- and T_3-induced changes in total cytochrome P450 content and NADPH-cytochrome P450 reductase activity were paralleled by a significant increase in the microsomal NADPH-dependent $O_2^{\cdot-}$ generation, expressed either per mg of protein (92 and 53%, respectively) or per nmol of cytochrome P450 (35 and 150%, respectively) (Fig. 2A). Considering that HCCH and T_3 elicited a 17 and 37% reduction in the activity of cytosolic SOD (Fig. 2B), the respective $O_2^{\cdot-}$ generation/SOD activity ratios were enhanced by 135% over control values (Fig. 2C). This unbalance in the $O_2^{\cdot-}$ production/utilization equilibrium by HCCH or T_3 treatment indicates a major derangement in the disposition of the free radical, that may contribute to the enhancement in the oxidative stress status of the liver (Videla et al., 1990; Videla, 2000). The above findings, and the observation that the activity of the NADPH-generating enzyme glucose-6-phosphate dehydrogenase is not altered by HCCH (Junqueira et al., 1986) or increased by T_3 (Simon-Giavarotti et al., 1998), may imply greater rates of cytochrome P450 reduction and, consequently, of $O_2^{\cdot-}$ production in both experimental conditions (Fig. 2A). This suggestion is supported by the significant enhancement elicited by HCCH and T_3 administration upon liver microsomal NADPH-dependent O_2 consumption

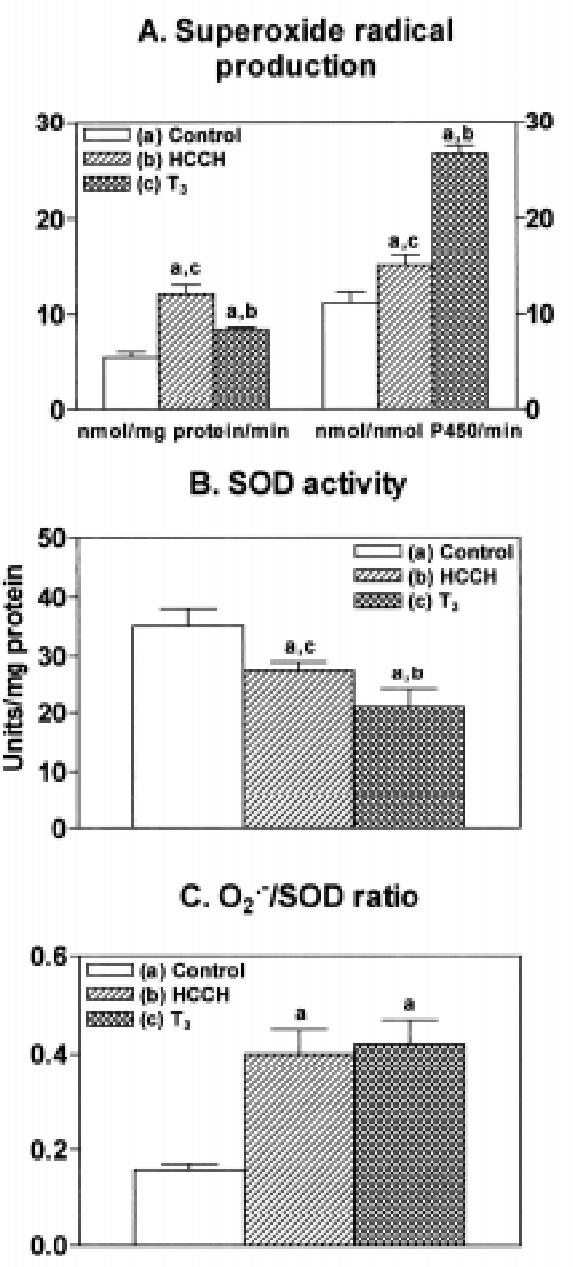

Figure 2. Microsomal NADPH-dependent superoxide radical ($O_2^{\cdot-}$) production (A), superoxide dismutase (SOD) activity (B), and $O_2^{\cdot-}$ production/SOD activity ratios (C) in the liver of control rats and animals subjected to γ-hexachlorocyclohexane (HCCH) or L-3,3',5-triiodothyronine (T_3) administration. Control rats for HCCH and T_3 treatments exhibited comparable values ($p>0.05$) and constitute a single group. Values shown correspond to means ± SE for 7-13 animals per experimental group. The significance of the differences between mean values ($P< 0.05$) was assessed by one-way ANOVA and the Newman-Keuls' test, and it is shown by the letters identifying each experimental group.

(Videla et al., 1988; Fernández et al., 1988), which is largely inhibited by the free radical scavenger cyanidadol, and in NADPH oxidase activity (Fernández et al., 1985; Junqueira et al., 1994). In addition to these changes, HCCH and T_3 also induce liver microsomal chemiluminescence, a response related to the formation of electronically excited species in oxidative radical reactions (Fernández et al., 1985; Junqueira et al. 1988), thus indicating an overall pro-oxidant condition at the microsomal level.

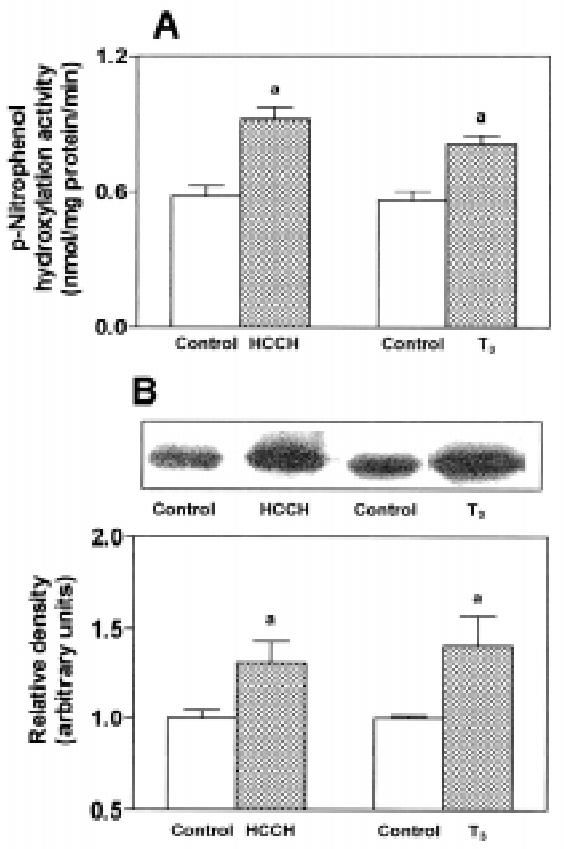

Figure 3. Microsomal activity of p-nitrophenol hydroxylation (A) and western blot analysis of cytochrome P4502E1 (B) in the liver of control rats and animals subjected to γ-hexachlorocyclohexane (HCCH) or L-3,3',5-triiodothyronine (T_3) administration. Representative blots shown in the upper panel in B contain 30 μg of microsomal protein from a different rat per lane. Data on the lower panel in B show the densitometric analysis of the immunoblots presented in the upper panel; for this purpose, data from both groups of control rats were set to unity, and values from HCCH- and T_3-treated rats were normalized to respective control group. Values shown correspond to means ± SE for 4-10 animals per group in A and 3 rats per group in B. The significance of the differences between mean values ($P < 0.05$) was assessed by one-way ANOVA and the Newman-Keuls test, and it is shown by the letters identifying each experimental group.

Assessment of the rate of hydroxylation of PNP by liver microsomes showed increases of 58 and 45% in HCCH- and T_3-treated rats, respectively (Fig. 3A), with the parallel induction of cytochrome P4502E1 (Fig. 3B). The significant 31% (HCCH) and 41% (T_3) increases in the content of cytochrome P4502E1 support the latter finding, assessed by western immunoblotting (Fig. 3B). Considering that microsomal PNP hydroxylation depends on the content of cytochrome P4502E1 (Reinke and Moyer, 1985; Koop, 1986), these results suggest the possibility that induction of cytochrome P4502E1 by HCCH and T_3 may play a role in the increased production of $O_2^{\cdot-}$. This hypothesis is supported by the substantial NADPH oxidase activity exhibited by cytochrome P4502E1 when reduced by NADPH-cytochrome P450 reductase (Gorsky et al., 1984; Ingelman-Sundberg and Johanson, 1984), and by the significant correlation established between microsomal $O_2^{\cdot-}$ generation and PNP hydroxylation ($r = 0.96$; $p<0.01$) or cytochrome P4502E1 content ($r=0.68$; $p<0.05$). In agreement with these findings, induction of cytochrome P4502E1 exhibits a significant correlation with lipid peroxidation, production of reactive O_2 species, formation of 1-hydroxylethyl radical from ethanol, and liver injury after prolonged ethanol feeding (Ekström and Ingelman-Sundberg, 1989; Reinke et al., 1990). In humans, cytochrome P4502E1 induction has been proposed to play a key role in the production of cellular injury in alcoholic hepatitis (Takahashi et al., 1993), a feature observed also in patients with nonalcoholic fatty liver disease (Weltman et al., 1998). Furthermore, significant cytotoxicity is observed in a transfected HepG2 cell line expressing human cytochrome P4502E1 when exposed to acetaminophen or ethanol (Dai and Cederbaum, 1995; Wu and Cederbaum, 1996).

Collectively, data presented indicate that HCCH and T_3 enhance the expression and activity of cytochrome P4502E1 in rat liver. These effects occur regardless of the changes in total cytochrome P450 content, involve a substantial increase in the

molecular activity of NADPH-cytochrome P450 reductase of liver microsomes, and may represent contributory mechanisms for NADPH-dependent $O_2^{\cdot-}$ generation and related hepatotoxicity (Videla et al., 1990; Videla, 2000). The latter view is in line with the finding that enhanced susceptibility of the liver to the toxic effects of acute lindane treatment in hyperthyroid state is produced by potentiation of the hepatic oxidative stress status (Videla et al., 1995; Simon-Giavarotti et al., 1998). This effect involves increases in the molecular activity of NADPH-cytochrome P450 reductase and in the $O_2^{\cdot-}$/SOD ratio, and reduction of hepatic α-tocopherol and GSH contents, in a magnitude exceeding the sum of effects elicited by the separate treatments (Videla et al., 1995; Simon-Giavarotti et al., 1998).

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