ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 2, 2004

Original Research Communication

Effects of Acute γ-Hexachlorocyclohexane Intoxication in Relation to the Redox Regulation of Nuclear Factor-κB, Cytokine Gene Expression, and Liver Injury in the Rat

LUIS A. VIDELA,[1] GLADYS TAPIA,[1] PATRICIA VARELA,[2] PAMELA CORNEJO,[1] JULIA GUERRERO,[3] YEDY ISRAEL,[4] and VIRGINIA FERNÁNDEZ[1]

ABSTRACT

γ-Hexachlorocyclohexane-induced hepatotoxicity is associated with oxidative stress. We tested the hypothesis that γ-hexachlorocyclohexane triggers the redox activation of nuclear factor-κB (NF-κB), leading to proinflammatory cytokine expression. Liver NF-κB activation (electrophoretic mobility shift assay), tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) mRNA expression (reverse transcription–polymerase chain reaction), and their serum levels (enzyme-linked immunosorbent assay) were measured at different times after γ-hexachlorocyclohexane treatment (50 mg/kg). The relationship between these and hepatic O_2 uptake, glutathione and protein carbonyl levels, and sinusoidal lactate dehydrogenase (LDH) efflux in liver perfusion studies was determined. γ-Hexachlorocyclohexane increased liver NF-κB DNA binding at 14–22 h after treatment, concomitantly with significant glutathione depletion and an increase in the rate of O_2 consumption, the content of protein carbonyls, and the sinusoidal LDH efflux. In these conditions, the expression of TNF-α and IL-1α is enhanced, with maximal increases in their respective mRNA content and serum levels of the cytokines being elicited at 18 h after γ-hexachlorocyclohexane treatment. All these changes are suppressed by the administration of α-tocopherol (100 mg/kg) or the Kupffer cell inactivator gadolinium chloride (10 mg/kg) prior to γ-hexachlorocyclohexane. γ-Hexachlorocyclohexane-induced TNF-α levels in serum are suppressed by pretreatment with an antisense oligonucleotide (ASO TJU-2755; daily doses of 10 mg/kg for 2 days) targeting the primary transcript for the cytokine, whereas those of IL-1α are not modified. It is concluded that γ-hexachlorocyclohexane-induced liver oxidative stress triggers the DNA binding activity of NF-κB, with the consequent increase in the expression of NF-κB-dependent genes for TNF-α and for IL-1α, factors that may mediate the hepatotoxicity of the insecticide. *Antioxid. Redox Signal.* 6, 471–480.

INTRODUCTION

γ-HEXACHLOROCYCLOHEXANE (lindane) is an important pesticide extensively used for public health and agricultural purposes in developing countries, which induces toxicity in several tissues, including the liver. γ-Hexachlorocyclohexane-induced hepatotoxicity is associated with the production of reactive species, including (a) electrophilic γ-hexachlorocyclohexane-derived metabolites generated during the biotransformation of the insecticide by cytochrome P450 (2, 45), (b)

[1]Molecular and Clinical Pharmacology Programme and [2]Cellular and Molecular Biology Programme, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile.
[3]Intensive Care Unit, Department of Internal Medicine, University of Chile Clinical Hospital, Santiago, Chile.
[4]Gene Therapy Laboratory, Millennium Institute in Cell Biology and Biotechnology, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santiago, Chile.

reactive O_2 intermediates, such as superoxide radical due to cytochrome P450 induction (19, 20), and (c) reactive nitrogen species, such as nitric oxide (9). Enhancement of nonspecific free radical formation, measured by chemiluminescence, and superoxide radical generation is also elicited in hepatic microsomes and mitochondria upon addition of γ-hexachlorocyclohexane *in vitro* (1). γ-Hexachlorocyclohexane-induced free radical activity leads to depletion of hepatic antioxidant stores, including reduced glutathione (GSH) (4) and lycopene (34) levels, and the inactivation of superoxide dismutase and catalase (19). Thus, disruption of the steady-state balance between generation of prooxidant species and their utilization in favor of the prooxidants is established in the liver (45), with the consequent enhancement in lipid peroxidation (1, 14, 19, 20, 31, 48) and protein oxidation (9) indices. In agreement with these findings, γ-hexachlorocyclohexane administration to rats increases the total O_2 consumption by the liver, an effect that involves both O_2 equivalents related to oxidative stress (44) and uncoupling of mitochondrial oxidative phosphorylation by lindane-derived chlorophenol metabolites (15). Development of oxidative stress by γ-hexachlorocyclohexane coincides with the onset and progression of liver injury (20). These features are readily reversible depending on the hepatic content of the insecticide and on the extent of microsomal superoxide generation (21), and are triggered in different cell types (1, 13, 28) and tissues (27, 32, 37) of experimental animals, plants (36), and also evidenced in the blood from poisoning human cases (3).

Current evidence indicates that oxidative stress is implicated in the genesis of cell injury through redox regulation of the transcription of genes involved in the pathogenesis of diseases (33, 40), in addition to direct mechanisms determining the oxidative damage to essential biomolecules (45). Nuclear factor-κB (NF-κB) is a prototype redox-sensitive transcription factor having a prominent role in the regulation of immune and inflammatory genes, apoptosis, and cell proliferation (18). Activation of NF-κB in Kupffer cells is important in the homeostatic response to acute or mild liver injury or as a pathological event underlying chronic liver disease (40). Therefore, the aim of present study was to test the hypothesis that γ-hexachlorocyclohexane-induced oxidative stress in the liver activates NF-κB, triggering the expression of NF-κB-dependent gene for tumor necrosis factor-α (TNF-α) and for interleukin-lα (IL-1α) at the Kupffer cell level. For this purpose, liver NF-κB DNA binding, hepatic TNF-α mRNA and IL-1α mRNA contents, and serum TNF-α and IL-1α levels were determined in control rats and in animals administered γ-hexachlorocyclohexane at different times after treatment. Separate groups of control and γ-hexachlorocyclohexane-treated rats were pretreated with the antioxidant α-tocopherol or the Kupffer cell inactivator gadolinium chloride ($GdCl_3$) (17), and results obtained were correlated with changes in biochemical parameters related to oxidative stress and liver injury.

MATERIALS AND METHODS

Animals and treatments

Male Sprague–Dawley rats (Bioterio Central, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile) weighing 200–300 g were housed on a 12-h light/dark cycle and were provided rat chow (Champion S.A., Santiago, Chile) and water *ad libitum*. Animals received a single intraperitoneal injection of 50 mg of γ-hexachlorocyclohexane/kg or an equivalent volume of vehicle (corn oil; controls), and studies were carried out at the indicated times after treatment. Separate groups of rats were subjected to either (a) 100 mg of α-tocopherol/kg i.p. 17 h prior to γ-hexachlorocyclohexane or (b) 10 mg of $GdCl_3$/kg i.v. 24 h before γ-hexachlorocyclohexane, and studies were performed at the indicated times after γ-hexachlorocyclohexane treatment. Animals used were cared according to the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (NIH publication no. 86-23).

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared on ice from liver homogenates as described by Deryckere and Gannon (10). In brief, liver homogenates were centrifuged for 30 s at 2,000 rpm and 4°C, and the supernatant was incubated for 5 min and centrifuged for 5 min at 5,000 rpm and 4°C. The pellet was resuspended in a buffer containing 20 m*M* HEPES (pH 7.9), 25% glycerol, 420 m*M* NaCl, 1.2 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, 0.5 m*M* phenylmethylsulfonyl fluoride, 2 m*M* benzamidine, and 5 μg/ml of the protease inhibitors pepstatin, leupeptin, and aprotinin, incubated on ice for 20 min, and centrifuged for 30 s at 13,000 rpm and 4°C. The supernatant was stored at −80°C. Protein concentration was determined according to Bradford (6). The samples were subjected to EMSA to assess the amount of active NF-κB involved in protein–DNA interactions (49), using labeled and unlabeled oligonucleotides containing the consensus sequence 5′-GATCTCAGAGGGGACTTTCCGAG-3′ (Genset Corp., La Jolla, CA, U.S.A.). The specificity of protein binding in nuclear extracts was confirmed in competition experiments using 100-fold excess of unlabeled probe. To assess the subunit composition of DNA binding protein, specific antibodies were used for supershift assay (43) (goat and rabbit immunoglobulin G raised against NF-κB p50 and p65, respectively; Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Samples were loaded on nondenaturating 6% polyacrylamide gels and run until the free probe reached the end of the gel; NF-κB bands were detected by autoradiography and quantified by densitometry using Scion Image (Scion Corp., Frederick, MD, U.S.A.).

Isolation of hepatic RNA and reverse transcription–polymerase chain reaction (RT-PCR) assay for cytokine mRNA

Total cellular RNA was extracted from liver samples from each rat with TRIzol reagent (GibcoBRL, Rockville, MD, U.S.A.) (8) and quantified by measurement of ultraviolet absorption at 260 nm. For RT-PCR assay of mRNA, first-strand cDNA was synthesized from total RNA using SuperScript RNase H− Reverse Transcriptase (Invitrogen Corp., Rockville, MD, U.S.A.) and random hexamer primers [$pd(N)_6$] (Promega, Madison, WI, U.S.A.). cDNA was amplified in a PCR reaction using Taq DNA polymerase (Invitrogen Corp.) in the presence of primers specific for rat cytokines. Nucleotide sequences for

sense and antisense primers used were 5′-CACGCTCTTCTGTCTACTGA-3′ and 5′-GGACTCCGTGATGTCTAAGT-3′ for TNF-α, and 5′-ATGGCCAAAGTTCCTGACTTGTTT-3′ and 5′-TTCATGATGAACTCCTGCTTG-3′ for IL-1α (Invitrogen Corp.). Thermocycling conditions for PCR were defined for each cytokine. RNA concentrations and PCR cycler were titrated to establish standard curves to document linearity and to allow semiquantitative analysis of signal strength. To control the relative amount of total mRNA transcribed in each reverse transcriptase reaction, RNA 18S invariant standards [Classic 18S Internal Standards (489 bp) or Classic II 18S Standards (324 bp); Ambion, The RNA Co., Austin, TX, U.S.A.] were used. PCR products were electrophoresedon 2% agarose (Invitrogen Corp.). The gels were stained with ethidium bromide and analyzed by densitometry using Scion Image (Scion Corp.).

Enzyme-linked immunoabsorbent assay (ELISA) for cytokines

Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and serum from blood obtained by cardiac puncture was separated and stored at −80°C. Levels of serum TNF-α (UltraSensitive Cytoscreen™ KRC3013 kit, Biosource International, Camarillo, CA, U.S.A.) and IL-1α (Endogen Inc., Woburn, MA, U.S.A.) were measured by ELISA according to the manufacturers' specifications. Serum samples containing high cytokine levels were repeated after dilution to assure assay results within the standard curve.

Parameters related to oxidative stress and liver cell injury

Livers were perfused via the portal vein in a nonrecirculating system as described previously (44), using Krebs–Henseleit bicarbonate buffer saturated with 95% O_2/5% CO_2 to give pH 7.4, at constant flow rates (3.5–4.0 ml/g of liver/min) and temperature (36–37°C). Livers were allowed to equilibrate for 15 min, and perfusate samples were taken every 5 min for 15 min to measure lactate dehydrogenase (LDH) activity (one unit corresponds to 1 μmol/min at 37°C), with the parallel and continuous polarographic measurement of O_2 consumption (44). At the end of perfusion, LDH activity was assessed in the tissue (U/g of liver) (5), concomitantly with the content of total glutathione (GSH) equivalents (38) and that of protein carbonyls as index of protein oxidation (29). Rates of sinusoidal LDH efflux (U/g of liver/min), as a liver cell viability parameter, were calculated from the perfusate activity and the flow rate, integrated between 15 and 30 min of perfusion

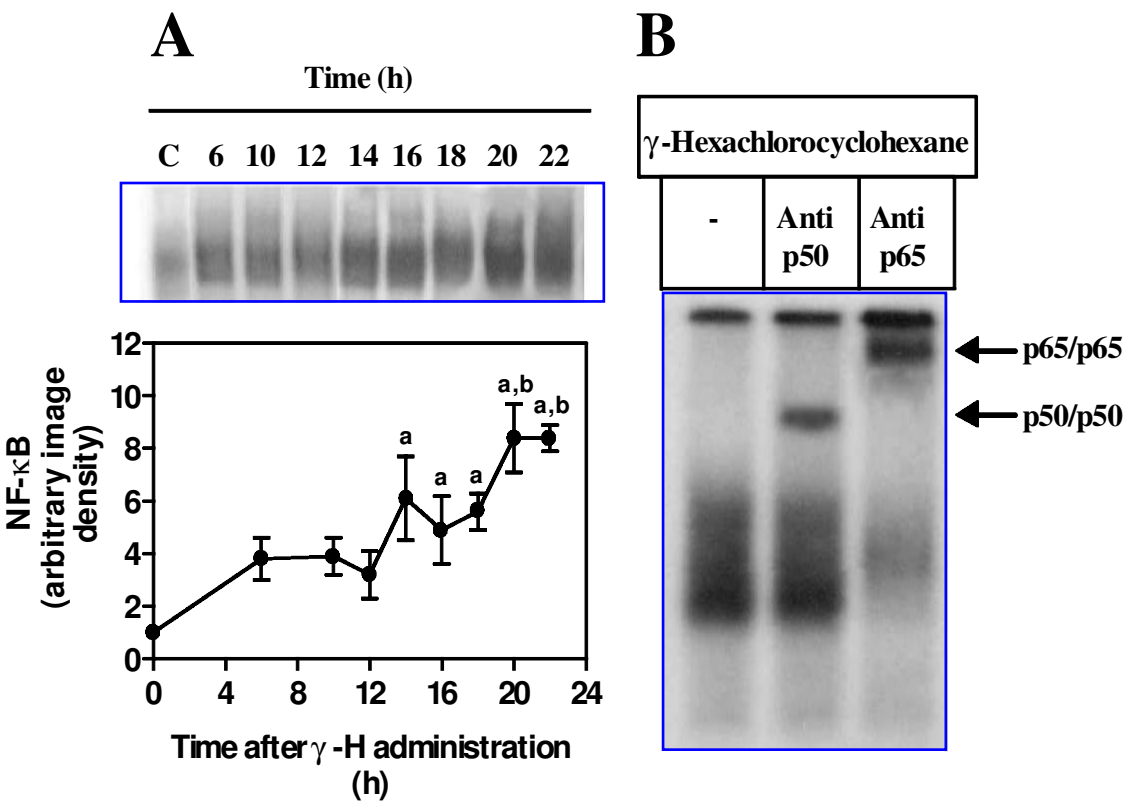

FIG. 1. Effect of acute γ-hexachlorocyclohexane (γ-H) administration on rat liver NF-κB DNA binding activity. (A) Representative autoradiograph of NF-κB DNA binding activity evaluated by EMSA using nuclear extracts from livers of control rats (C, time zero) and γ-H-treated animals (50 mg/kg) at different times after treatment, and the respective densitometric quantification of relative activity of NF-κB. Mean value at time zero was arbitrarily set to unity, and values at other time points were normalized to this. Data are means ± SEM for three to six different animals. Significance studies: $^{a}p < 0.05$ versus controls at time zero; $^{b}p < 0.05$ versus γ-H-treated rats at 6, 10, and 12 h after treatment. **(B)** Supershift analysis for assessment of the composition of NF-κB induced at 20 h after γ-H treatment.

(U/g of liver), and expressed as percentage of the hepatic LDH activity (fractional LDH efflux).

Chemicals and reagents used were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), except for those stated in the above sections.

Statistical analysis

Values shown are means ± SEM for the number of separate experiments indicated. The statistical significance of differences in mean values among multiple groups was made by one-way ANOVA and the Student–Newman–Keuls post-hoc test. In all cases, a value of $p < 0.05$ was considered significant.

RESULTS

γ-Hexachlorocyclohexane administration increases rat liver NF-κB DNA binding activity. Suppression by α-tocopherol or $GdCl_3$ pretreatment

NF-κB DNA binding activity exhibited a progressive enhancement following γ-hexachlorocyclohexane administration, with significant increases starting at 14 h after γ-hexachlorocyclohexane treatment and maximal effects being observed at 20–22 h (Fig. 1A). Supershift analysis using antibodies specific for NF-κB p50 (anti p50) or p65 (anti p65) disrupted the complexes induced at 20 h after γ-hexachlorocyclohexane treatment, thus confirming the presence of NF-κB isoforms (Fig. 1B). Rats subjected to α-tocopherol or $GdCl_3$ exhibited liver NF-κB DNA binding activity comparable to that in control animals, whereas both pretreatments abolished the eightfold increase in NF-κB DNA binding activity induced by γ-hexachlorocyclohexane at 20 h after treatment (Fig. 2).

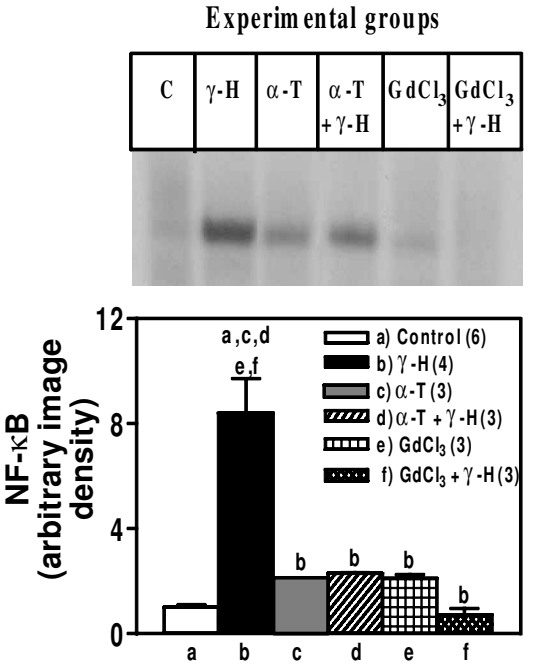

FIG. 2. Representative autoradiograph and densitometric quantification of relative activity of NF-κB in control and γ-hexachlorocyclohexane (γ-H)-treated animals (20 h) subjected to α-tocopherol (α-T) or $GdCl_3$ pretreatments. Data are means ± SEM for the number of rats indicated in parentheses, with significance assessment ($p < 0.05$) shown by the letters identifying each group.

Effects of γ-hexachlorocyclohexane administration on liver O_2 consumption, sinusoidal LDH efflux, and content of GSH and protein carbonyls. Modification by α-tocopherol or $GdCl_3$ pretreatment

The maximal enhancement of liver NF-κB DNA binding activity observed at 20 h after γ-hexachlorocyclohexanetreatment is accompanied by significant increases in the basal rate of O_2 consumption of the liver (21%; Fig. 3A) and in the sinusoidal fractional efflux of LDH (356%; Fig. 3B), concomitantly with a 24% reduction in the content of hepatic GSH (Fig. 3C) and a 355% elevation in protein carbonyls as index of protein oxidation (Fig. 3D). These γ-hexachlorocyclohexane-inducedchanges are suppressed in γ-hexachlorocyclohexane-treated rats pretreated with the antioxidant α-tocopherol or the Kupffer cell inactivator $GdCl_3$ (Fig. 3).

γ-Hexachlorocyclohexane administration promotes liver TNF-α and IL-1α expression

γ-Hexachlorocyclohexane-induced liver NF-κB DNA binding activity (Fig. 1A) was paralleled by increases in hepatic TNF-α expression, evidenced by a significant enhancement in the levels of TNF-α mRNA in the 14–18-h time interval after γ-hexachlorocyclohexane treatment, returning toward control values at later times (Fig. 4A). At 18 h after γ-hexachlorocyclohexane administration, the increase in hepatic TNF-α mRNA levels was abolished by pretreatment with either α-tocopherol or $GdCl_3$ (Fig. 4B). γ-Hexachlorocyclohexane administration also stimulated the hepatic expression of IL-1α, a response that is observed in the 14–22-h time interval, with a maximal effect being found at 18 h (Fig. 4C). At this latter experimental time, enhancement in hepatic IL-1α mRNA levels by γ-hexachlorocyclohexane was suppressed by both α-tocopherol and $GdCl_3$ pretreatments (Fig. 4D). In these conditions, γ-hexachlorocydohexane elicited significant increases in the serum levels of TNF-α (Fig. 5A) and IL-1α (Fig. 6A) in the 14–20-h time interval, with maximal effects at 18 h after treatment being abolished by both α-tocopherol and $GdCl_3$ pretreatments (Fig. 5B and 6B). In addition, pretreatment with the antisense oligonucleotide targeting the primary transcript of TNF-α ASO TJU-2755 (daily doses of 10 mg/kg i.v. for two consecutive days, 24 h prior to γ-hexachlorocyclohexane) (42) suppressed γ-hexachlorocyclohexane-inducedserum TNF-α enhancement at 18 h after treatment (Fig. 5B), without significantly affecting the increase elicited in IL-1α levels (Fig. 6B).

DISCUSSION

Data reported in this study demonstrate that the administration of a single dose of γ-hexachlorocyclohexane to rats

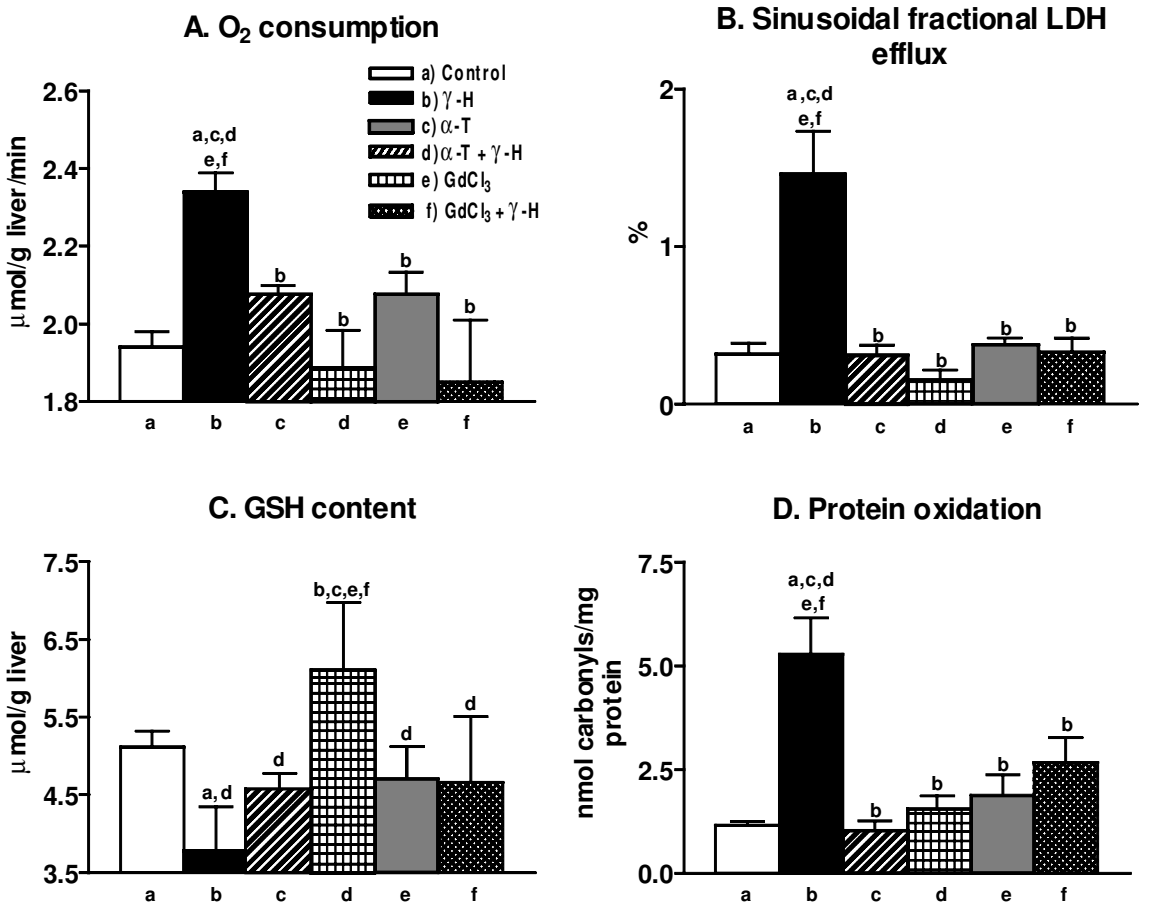

FIG. 3. Effect of pretreatment with α-tocopherol (α-T) and $GdCl_3$ on γ-hexachlorocyclohexane (γ-H)-induced changes in hepatic (A) O_2 consumption rate, (B) sinusoidal fractional efflux of LDH, (C) total reduced GSH content, and (D) levels of protein carbonyls at 20 h after γ-H treatment in the rat. Data are means ± SEM for three to 11 different animals, with significance assessment ($p < 0.05$) shown by the letters identifying each group.

enhances the DNA binding activity of hepatic NF-κB, an effect that is related to the enhancement in the oxidative stress status of the liver. This view is in agreement with previous time-course studies showing that hepatic formation of thiobarbituric acid reactants, NADPH-supported chemiluminescence, and superoxide radical production/superoxide dismutase activity ratios are significantly increased at 6 h after γ-hexachlorocyclohexane administration and maximally enhanced at 24 h (20), which correlate with the maximal NF-κB activation found at 20–22 h (Fig. 1A). Furthermore, the increases in hepatic respiration and protein oxidation, GSH depletion, and activation of NF-κB observed at 20 h after γ-hexachlorocyclohexane treatment are abolished by α-tocopherol pretreatment, thus suggesting a cause–effect relationship between oxidative stress and activation of the transcription factor, although the role of mechanisms that are not dependent on the cellular redox status cannot be excluded. γ-Hexachlorocyclohexane-induced DNA binding activity of hepatic NF-κB occurs primarily at the Kupffer cell level, considering its suppression by $GdCl_3$ pretreatment. This rare earth metal eliminates large Kupffer cells (17), with the consequent reduction in the phagocytic (7, 46) and respiratory burst (46) activities of liver macrophages. It is of interest that an interrelationship between the oxidative stress status and Kupffer cell activity appears to exist, evidenced by the inhibition of γ-hexachlorocyclohexane-induced liver protein oxidation, lipid peroxidation (35), and GSH depletion by $GdCl_3$. The suppression of γ-hexachlorocyclohexane-induced O_2 consumption by $GdCl_3$ and α-tocopherol suggests that significant mitochondrial uncoupling is not achieved in these conditions, as reported in isolated rat liver mitochondria (15), and indicates O_2 equivalents used at the Kupffer cell level that may involve O_2 utilization for production of reactive oxygen species involved in NF-κB activation in these cells. In this respect, O_2 utilization by liver macrophage NADPH oxidase may play a role in γ-hexachlorocyclohexane-induced $GdCl_3$-sensitive respiration, providing a sufficient prooxidant status to induce NF-κB activation (40, 47). The role of NADPH oxidase in NF-κB activation underlies hydrogen peroxide generation in the respiratory burst of macrophages (22) and is supported by studies with the NADPH oxidase inhibitor diphenyleneiodonium sulfate (23) or by deletion of $p47^{phox}$, the regulatory component of NADPH oxidase (40), in an animal model of alcoholic liver injury. In addition to liver macrophage NADPH oxidase, Kupffer-cell cytochrome P4502E1 could also play a

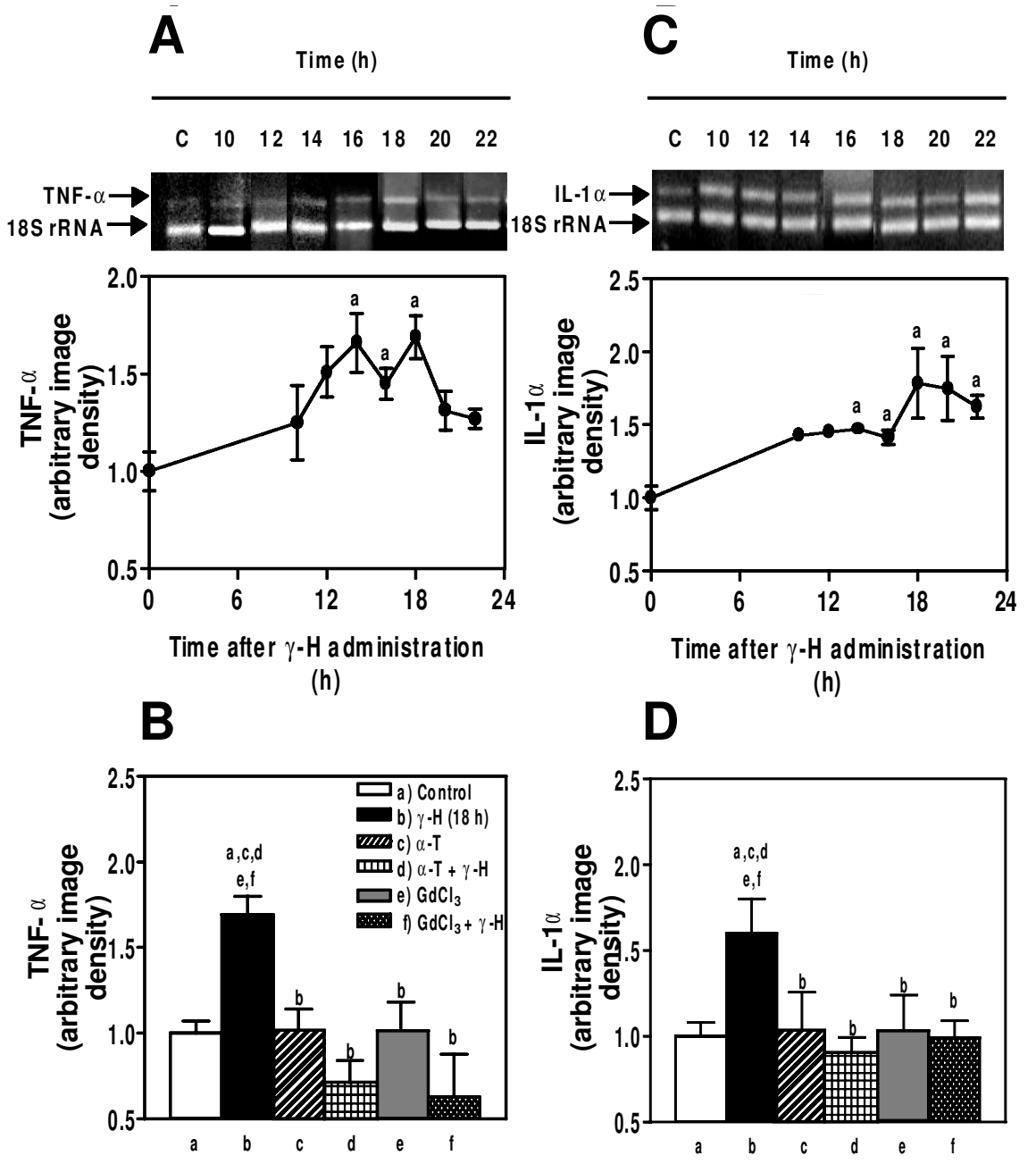

FIG. 4. Effects of acute γ-hexachlorocyclohexane (γ-H) administration on the liver expression of TNF-α and IL-1α. (A) Representative agarose gel electrophoresis of the RT-PCR products for TNF-α mRNA (580 bp) and 18S rRNA (489 bp) after ethidium bromide staining in total hepatic RNA samples from control rats (C, time zero) and γ-H-treated animals at different times after treatment, and the respective densitometric quantification of RT-PCR products of the mRNA of TNF-α expressed as the TNF-α/18S rRNA ratios to compare lane–lane equivalency in total RNA content. Mean value at time zero was arbitrarily set to unity, and values at other time points were normalized to this. **(B)** Influence of α-tocopherol (α-T) and $GdCl_3$ pretreatment on γ-H-induced TNF-α mRNA levels at 18 h after treatment. **(C)** Agarose gel electrophoresis of the RT-PCR products of IL-1α mRNA (408 bp) and 18S rRNA (324 bp) in total hepatic RNA samples from controls (C, time zero) and γ-H-treated rats at different times after treatment, and the respective densitometric quantification of the mRNA of IL-1α expressed as the IL-1α/18S rRNA ratios. **(D)** Effects of α-T and $GdCl_3$ pretreatment on γ-H-induced IL-1α mRNA levels at 18 h after treatment. Data are means ± SEM for three different rats. Significance studies; $^a p < 0.05$ compared with control values at time zero in (A) and (C); significance in (B) and (D) is shown by the letters identifying each experimental group ($p < 0.05$).

role in NF-κB activation by γ-hexachlorocyclohexane, considering that the pesticide enhances its expression and activity in rat liver (12). Cytochrome P4502E1 is characterized by a substantial prooxidant activity, which generates superoxide radical and hydrogen peroxide, and by its induction by chronic ethanol consumption in rat Kupffer cells (25). However, the

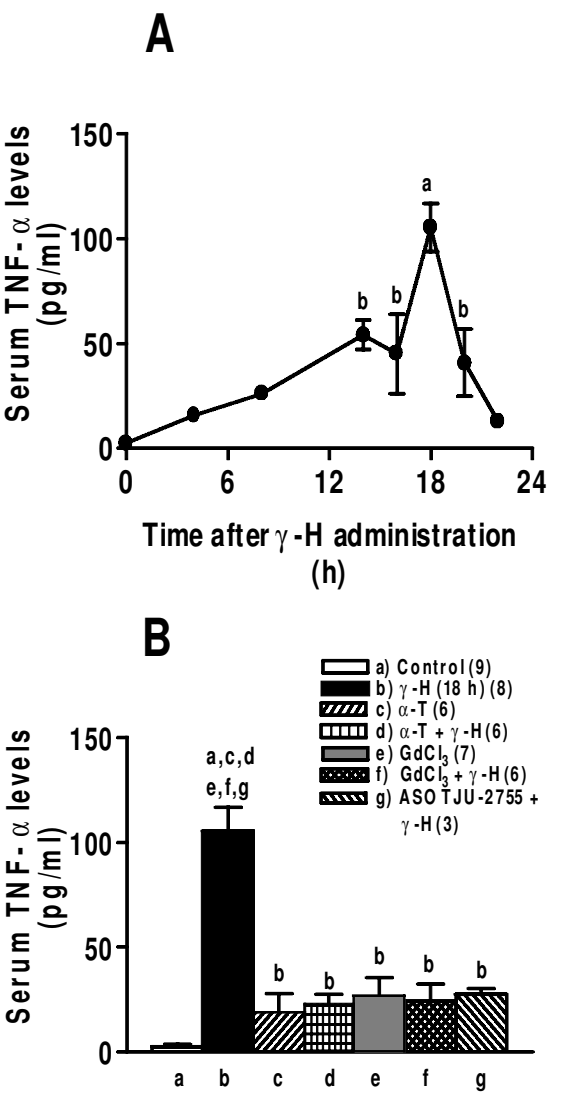

FIG. 5. Effect of acute γ-hexachlorocyclohexane (γ-H) administration on the serum levels of TNF-α at different times after treatment (A) and the influence of pretreatment with α-tocopherol (α-T), $GdCl_3$, and the antisense oligonucleotide targeting the primary transcript of TNF-α (ASO TJU-2755) assessed at 18 h after γ-hexachlorocyclohexane treatment (B). The serum levels of TNF-α were determined by ELISA. Data are means ± SEM for three to nine different animals. Significance studies in (A): $^{a}p < 0.05$ versus controls (time zero) and γ-H-treated rats at 4, 8, 14, 16, 20, and 22 h after treatment, and $^{b}p < 0.05$ versus controls and γ-H-treated rats at 18 h. Significance in (B) is shown by the letters identifying each group ($p < 0.05$).

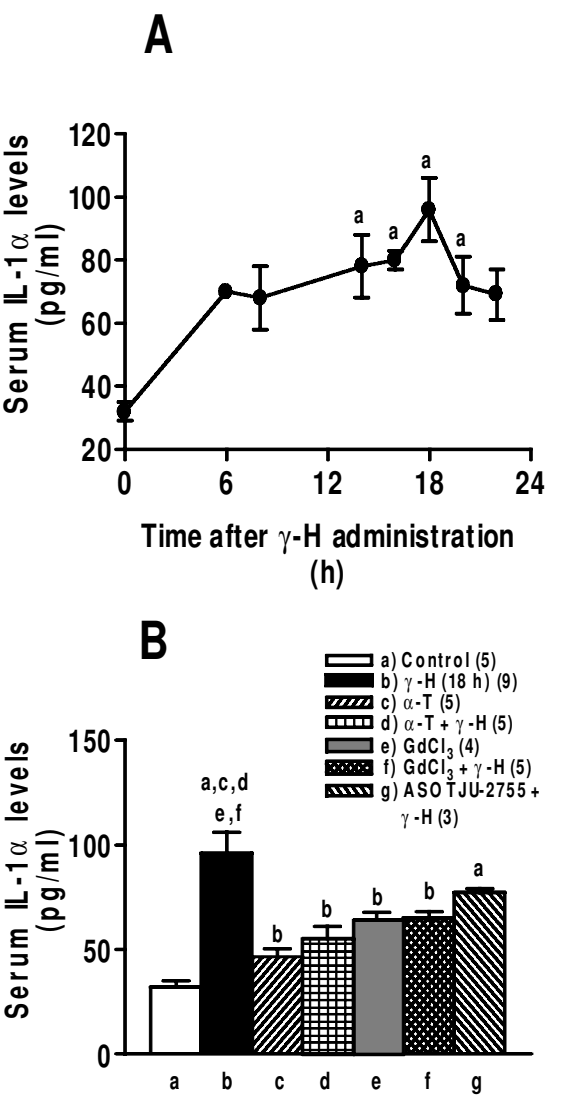

FIG. 6. Effect of acute γ-hexachlorocyclohexane (γ-H) administration on the serum levels of IL-1α at different times after treatment (A) and the influence of pretreatment with α-tocopherol (α-T), $GdCl_3$, and the antisense oligonucleotide targeting the primary transcript of TNF-α (ASO TJU-2755) assessed at 18 h after γ-H treatment (B). The serum levels of IL-1α were determined by ELISA. Data are means ± SEM for three to nine different animals. Significance studies in (A): $^{a}p < 0.05$ versus controls at time zero. Significance in (B) is shown by the letters identifying each group ($p < 0.05$).

role of cytochrome P4502E1 and that of aldehydic products arising from γ-hexachlorocyclohexane-induced lipid peroxidation and/or reactive γ-hexachlorocyclohexane-derivedmetabolites in NF-κB activation in Kupffer cells remain to be established.

In these conditions, livers from animals subjected to γ-hexachlorocyclohexane administration with increased NF-κB DNA binding activity concomitantly exhibit mRNA expression of the NF-κB responsive gene for TNF-α. In addition, the expression of the IL-1α gene is also up-regulated by γ-hexachlorocyclohexane, a process that is triggered by several signals, including TNF-α (11). Thus, induction of the expression of IL-1α in the liver of γ-hexachlorocyclohexane-treated rats might also result from the up-regulation of the TNF-α gene through the redox activation of NF-κB, leading to an enhancement in the serum levels of both cytokines sharing similar biologic activities. As the increases in IL-1α induced by γ-hexachlorocyclohexane are not significantly reduced by an antisense oligonucleotide that fully blocked the γ-hexachloro-

cyclohexane-induced TNF-α enhancement, these data suggest that the increases in both cytokines are mediated by a third common factor, likely the activation of the NF-κB system, that is suppressed by pretreatment with α-tocopherol and $GdCl_3$.

Although Kupffer cells play a central role in the homeostatic response to liver injury with release of TNF-α and IL-1α, these mediators can be key effector molecules for induction of hepatotoxicity when their expression is sustained or enhanced, or the target cells become sensitized (40). Morphological evidence shows that hepatic cell injury following acute γ-hexachlorocyclohexane intoxication is fully developed at 24 h after treatment (20) and regresses to normal from the third day and thereafter (21). Liver injury observed at 20 h after γ-hexachlorocyclohexane administration, assessed by the substantial elevation in the sinusoidal release of LDH during perfusion studies, coincides with the higher levels of TNF-α and IL-1α in serum, which are sustained from 14 h to 20 h. Moreover, γ-hexachlorocyclohexane-induced liver LDH release is suppressed by α-tocopherol and $GdCl_3$ pretreatments, thus supporting the interrelationship between the redox activation of hepatic NF-κB, the up-regulation of TNF-α and IL-1α expression, and hepatotoxicity. This view is in agreement with data showing that the free radical-mediated redox activation of transcription factors (NF-κB and/or activator protein-1) transactivating promoters of inflammatory genes plays a central role in the hepatotoxicity of carbon tetrachloride (7, 16, 26), ethanol (23, 40), or acetaminophen (24). γ-Hexachlorocyclohexane-induced liver oxidative stress and hepatocellular injury are readily reversible (21), a phenomenon that may involve resetting of gene expression at the Kupffer cell level to favor cell survival and reparative responses.

In summary, data presented support the contention that acute γ-hexachlorocyclohexane administration triggers the DNA binding activity of hepatic NF-κB by a redox mechanism exerted at the Kupffer cell level, with the consequent up-regulation of TNF-α and IL-1α expression and related hepatotoxicity. Liver oxidative stress-dependent NF-κB activation occurs in the 14–22-h time period after *in vivo* γ-hexachlorocyclohexane treatment, although NF-κB is an acute transcription factor (41) that is activated as early as 10 min by the respiratory burst of carbon-stimulated Kupffer cells in liver perfusion studies (30) or within 30 min in isolated Kupffer cells exposed to lipopolysaccharide (39).

ACKNOWLEDGMENTS

This work was supported by grant 1000887 from FONDECYT, Chile.

ABBREVIATIONS

ELISA, enzyme-linked immunoabsorbent assay; EMSA, electrophoretic mobility shift assay; $GdCl_3$, gadolinium chloride; GSH, reduced glutathione; IL-1α, interleukin-1α; LDH, lactate dehydrogenase; NF-κB, nuclear factor-κB; RT-PCR, reverse transcription–polymerase chain reaction; TNF-α, tumor necrosis factor-α.

REFERENCES

1. Bagchi M and Stohs SJ. In vitro induction of reactive oxygen species by 2,3,7,8-tetrachlorodibenzo*p*-dioxin, endrin, and lindane in rat peritoneal macrophages, and hepatic mitochondria and microsomes. *Free Radic Biol Med* 14: 11–18, 1993.
2. Baker MT, Nelson RM, and Van Dyke RA. The formation of chlorobenzene and benzene by the reductive metabolism of lindane by rat liver microsomes. *Arch Biochem Biophys* 236: 506–514, 1985.
3. Banerjee BD, Seth V, Bhattacharya A, Pasha ST, and Chakraborty AK. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. *Toxicol Lett* 107: 33–47, 1999.
4. Barros SBM, Videla LA, Simizu K, Van Halsema L, and Junqueira VBC. Lindane-induced oxidative stress. II. Time course of changes in hepatic glutathione status. *Xenobiotica* 18: 1305–1310, 1888.
5. Bergmeyer HU and Bernt E. Lactate dehydrogenase. In: *Methods of Enzymatic Analysis, Vol. 2*, edited by Bergmeyer HU. New York: Academic Press, 1974, pp. 574–579.
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
7. Camandola S, Aragno M, Cutrin JC, Tamagno E, Danni O, Chiarpotto E, Parola M, Leonarduzzi G, Biasi F, and Poli G. Liver AP-1 activation due to carbon tetrachloride is potentiated by 1,2-dibromoethane but is inhibited by α-tocopherol and gadolinium chloride. *Free Radic Biol Med* 26: 1108–1116, 1999.
8. Chomczynski P and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
9. Cornejo P, Tapia G, Puntarulo S, Galleano M, Videla LA, and Fernández V. Iron-induced changes in nitric oxide and superoxide radical generation in rat liver after lindane or thyroid hormone treatment. *Toxicol Lett* 119: 87–93, 2001.
10. Deryckere F and Gannon F. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques* 16: 405, 1994.
11. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerani A, Palladino MA, and O'Connor JV. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin-1. *J Exp Med* 163: 1433–1450, 1986.
12. Fernández V, Massa L, Quiñones L, Simon-Giavarotti KA, Giavarotti L, D'Almeida V, Azzalis LA, Junqueira VBC, and Videla LA. 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. *Biol Res* 36: 359–365, 2003.
13. Ginsburg I, Gibbs DF, Tarapchak S, and Varani J. A novel approach to the assessment of toxicity of hexachlorocyclohexane (lindane) and of certain organic solvents: killing of cells in culture and the release of arachidonate by sinergism among H_2O_2 membrane-damaging agents histone and trypsin. *In Vitro Toxicol* 9: 304–313, 1996.
14. Goel MR, Shara MA, and Stohs SJ. Induction of lipid peroxidation by hexachlorocyclohexane, dieldrin, TCDD, car-

bon tetrachloride, and hexachlorobenzene in rats. *Bull Environ Contam Toxicol* 40: 255–262, 1988.
15. Gopalaswamy UV and Alyar AS. Effects of lindane on liver mitochondrial function in the rat. *Bull Environ Contam Toxicol* 33: 106–113, 1984.
16. Gruebele A, Zawaski K, Kaplan D, and Novak RF. Cytochrome P4502E1- and cytochrome P4502B1/2B2-catalyzed carbon tetrachloride metabolism: effects on signal transduction as demonstrated by altered immediate-early (c-Fos and c-Jun) gene expression and nuclear AP-1 and NF-κB transcription factor levels. *Drug Metab Dispos* 24: 15–22, 1996.
17. Hardonk MJ, Dijkhuis FJ, Hulstaert CE, and Koudstaal J. Heterogeneity of rat and liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J Leukoc Biol* 52: 296–302, 1992.
18. Janssen-Heininger YMW, Poynter ME, and Baeuerle PA. Recent advances towards understanding redox mechanisms in the activation of nuclear factor κB. *Free Radic Biol Med* 28: 1317–1327, 2000.
19. Junqueira VBC, Simizu K, Videla LA, and Barros SBM. Dose-dependent study of the effects of acute lindane administration on rat liver superoxide anion production, antioxidant enzyme activities and lipid peroxidation. *Toxicology* 41: 193–204, 1986.
20. Junqueira VBC, Simizu K, Van Halsema L, Koch OR, Barros SBM, and Videla LA. Lindane-induced oxidative stress. I. Time-course of changes in hepatic microsomal parameters, antioxidant enzymes, lipid peroxidative indices, and morphological characteristics. *Xenobiotica* 18: 1297–1304, 1988.
21. Junqueira VBC, Koch OR, Arisi ACM, Fuzaro AP, Azzalis LA, Barros SBM, Cravero A, Farré S, and Videla LA. Regression of morphological alterations and oxidative stress-related parameters after acute lindane-induced hepatotoxicity in rats. *Toxicology* 117: 199–205, 1997.
22. Kaul N and Forman HJ. Activation of NFκB by the respiratory burst of macrophages. *Free Radic Biol Med* 21: 401–405, 1996.
23. Kono H, Rusyn I, Yin M, Gabele E, Yamashina S, Dikalova A, Kadiiska MB, Connor HD, Mason RP, Segal BH, Bradford BU, Holland SM, and Thurman RG. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *J Clin Invest* 106: 867–872, 2000.
24. Laskin DL and Laskin JD. Role of macrophages and inflammatory mediators in chemically induced toxicity. *Toxicology* 160: 11–118, 2001.
25. Lieber CS. Cytochrome P4502E1: its physiological and pathological role. *Physiol Rev* 77: 517–544, 1997.
26. Liu SL, Degli ES, Yao T, Diehl AM, and Zern MA. Vitamin E therapy of acute CCl_4-induced hepatic injury in mice is associated with inhibition of nuclear factor kappa B binding. *Hepatology* 22: 1474–1481, 1995.
27. McNutt TL and Harris C. Lindane embryotoxicity and differential alteration of cysteine and glutathione levels in rat embryos and visceral yolk sacs. *Reprod Toxicol* 8: 351–362, 1994.
28. Perocco P, Colacci A, Del Ciello C, and Grilli S. Cytotoxic and cell transforming effects of the insecticide, lindane (gamma-hexachlorocyclohexane)on BALB/c 3T3 cells. *Res Commun Mol Pathol Pharmacol* 89: 329–339, 1995.
29. Resnick AZ and Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol* 233: 357–363, 1994.
30. Romanque P, Tapia G, and Videla LA. Kupffer cell stimulation in the isolated perfused rat liver triggers nuclear factor-κB DNA binding activity. *Redox Rep* 8: 341–346, 2003.
31. Samanta L and Chainy GBN. Hexachlorocyclohexane-induced changes in lipid peroxidation, superoxide dismutase and catalase activities, and glutathione content in chick liver. *Ind J Exp Biol* 33: 131–133, 1995.
32. Samanta L and Chainy GBN. Response of testicular antioxidant enzymes to hexachlorocyclohexane is species specific. *Asian J Androl* 4: 191–194, 2002.
33. Sen CK and Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J* 10: 709–720, 1996.
34. Simon-Giavarotti KA, Rodrigues L, Rodrigues T, Junqueira VBC, and Videla LA. Liver microsomal parameters related to oxidative stress and antioxidant systems in hyperthyroid rats subjected to acute lindane treatment. *Free Radic Res* 29: 35–42, 1998.
35. Simon-Giavarotti KA, Giavarotti L, Gomes LF, Lima AF, Veridiano AM, García EA, Mora OA, Fernández V, Videla LA, and Junqueira VBC. Enhancement of lindane-induced liver oxidative stress and hepatotoxicity by thyroid hormone is reduced by gadolinium chloride. *Free Radic Res* 36: 1033–1039, 2002.
36. Sinha S. Oxidative stress induced by HCH in Hydrilla verticillata (l.f.) Royle: modulation in uptake and toxicity due to Fe. *Chemosphere* 46: 281–288, 2002.
37. Sujatha R, Chitra KC, Latchoumycandane C, and Mathur PP. Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. *Asian J Androl* 3: 135–138, 2001.
38. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: application to mammalian blood and other tissues. *Anal Biochem* 27: 502–522, 1969.
39. Tran-Thi TA, Decker K, and Baeuerle PA. Differential activation of transcription factors NF-kappa B and AP-1 in rat liver macrophages. *Hepatology* 22: 613–619, 1995.
40. Tsukamoto H. Redox regulation of cytokine expression in Kupffer cells. *Antioxid Redox Signal* 4: 741–748, 2002.
41. Tsukamoto H and Lin M. The role of Kupffer cells in liver injury. In: *Cells of the Hepatic Sinusoid, Vol. 6*, edited by Wisse E, Knook DL, and Balabaud C. Leiden, The Netherlands: The Kupffer Cell Foundation, 1997, pp. 244–250.
42. Tu G, Cao Q, Zhou F, and Israel Y. Tetranucleotide GGGA motif in primary RNA transcripts. Novel target site for antisense design. *J Biol Chem* 273: 25125–25131, 1998.
43. van Leeuwen HJ, van der Bruggen T, van Asbeck BS, and Boereboom FTJ. Effect of corticosteroids on nuclear factor-κB activation and hemodynamics in late septic shock. *Crit Care Med* 29: 1074–1077, 2001.
44. Videla LA, Simizu K, Barros SBM, and Junqueira VBC. Lindane-induced liver oxidative stress: respiratory alterations and the effect of desferrioxamine in the isolated perfused rat liver. *Cell Biochem Funct* 7: 179–183, 1989.

45. Videla LA, Barros SBM, and Junqueira VBC. Lindane-induced liver oxidative stress. *Free Radic Biol Med* 9: 169–179, 1990.
46. Videla LA, Troncoso P, Arisi ACM, and Junqueira VBC. Dose-dependent effects of acute lindane treatment on Kupffer cell function assessed in the isolated perfused rat liver. *Xenobiotica* 27: 747–757, 1997.
47. Wang J-F, Komarov P, and de Groot H. Luminol chemiluminescence in rat macrophages and granulocytes: the role of NO, O_2^-/H_2O_2, and HOCl. *Arch Biochem Biophys* 304: 189–196, 1993.
48. Yang FI and DiSilvestro RA. Lindane-induced rat liver lipid peroxidation without depressed Cu-Zn superoxide dismutase activities. *Pharmacol Toxicol* 70: 392–393, 1992.
49. Zabel U, Schreck R, and Baeuerle PA. DNA binding of purified transcription factor NF-κB. Affinity, specificity, Zn^{2+} dependence, and differential half-site recognition. *J Biol Chem* 266: 252–260, 1991.

Address reprint requests to:
Prof. Luis A. Videla
Programa de Farmacología Molecular y Clínica
ICBM
Facultad de Medicina
Universidad de Chile
Casilla 70000
Santiago-7, Chile

E-mail: lvidela@med.uchile.cl

Received for publication October 23, 2003; accepted December 12, 2003.

This article has been cited by:

1. Vesile Duzguner, Suat Erdogan. 2012. Chronic exposure to imidacloprid induces inflammation and oxidative stress in the liver & central nervous system of rats. *Pesticide Biochemistry and Physiology* **104**:1, 58-64. [CrossRef]
2. Nathalie Zucchini-Pascal, Georges de Sousa, Jerôme Pizzol, Roger Rahmani. 2011. Molecular investigation of the effects of lindane in rat hepatocytes: Microarray and mechanistic studies. *Food and Chemical Toxicology* . [CrossRef]
3. Rodrigo Castillo, Ramón Rodrigo, Felipe Perez, Mauricio Cereceda, René Asenjo, Jaime Zamorano, Roberto Navarrete, Eli Villalabeitia, Juan Sanz, Cristián Baeza, Rubén Aguayo. 2011. Antioxidant Therapy Reduces Oxidative and Inflammatory Tissue Damage in Patients Subjected to Cardiac Surgery with Extracorporeal Circulation. *Basic & Clinical Pharmacology & Toxicology* **108**:4, 256-262. [CrossRef]
4. D. Mladenovi#, D. Djuric, N. Petronijevi#, T. Radosavljevi#, N. Radonji#, D. Mati#, D. Hrn#i#, A. Raši#-Markovi#, D. Vu#evi#, D. Dekanski, O. Stanojlovi#. 2010. The correlation between lipid peroxidation in different brain regions and the severity of lindane-induced seizures in rats. *Molecular and Cellular Biochemistry* **333**:1-2, 243-250. [CrossRef]
5. Virginia Fernández, Solange Reyes, Sergio Bravo, Rodrigo Sepúlveda, Pamela Romanque, Gonzalo Santander, Iván Castillo, Patricia Varela, Gladys Tapia, Luis A. Videla. 2007. Involvement of Kupffer cell-dependent signaling in T₃-induced hepatocyte proliferation in vivo. *Biological Chemistry* **388**:8, 831. [CrossRef]
6. Howard P. GlauertVitamin E and NF##B Activation: A Review **76**, 135-153. [CrossRef]
7. Jason M. Hansen. 2006. Oxidative stress as a mechanism of teratogenesis. *Birth Defects Research Part C: Embryo Today: Reviews* **78**:4, 293-307. [CrossRef]
8. Gladys Tapia, Virginia Fernández, Cesar Pino, Rodrigo Ardiles, Luis A. Videla. 2006. The acute-phase response of the liver in relation to thyroid hormone-induced redox signaling. *Free Radical Biology and Medicine* **40**:9, 1628-1635. [CrossRef]