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Toxic Metals Stimulate Inflammatory Cytokines in Hepatocytes through Oxidative Stress Mechanisms

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Hepatocytes, as well as nonparenchymal cells, secrete proinflammatory cytokines and chemokines that are involved in the pathology of many liver diseases. In particular, tumor necrosis factor-α (TNFα), as well as members of the CXC family of chemokines, including interleukin (IL)-8 in humans and macrophage inflammatory protein (MIP)-2 in rodents, have been implicated in both damage and repair processes associated with various hepatotoxins. In the liver, cytokine secretion is usually associated with nonparenchymal cells, particularly Kupffer cells. In the present studies, cytokine gene expression and secretion were investigated in hepatocytes treated with cadmium chloride ($CdCl_2$) or vanadium pentoxide (V_2O_5). Using human Hep G2 cells and freshly isolated rodent hepatocytes, it was demonstrated that metals increase gene expression and secretion of CXC chemokines and TNFα. IL-8 and MIP-2 secretion induced either by the metals or H_2O_2 were inhibited by antioxidants such as tetramethyl-thiourea and *N*-acetyl-cysteine. In vitro neutralization experiments with TNFα and *in vivo* studies with TNFα receptor knockout mice indicated that the metals directly stimulate CXC chemokine secretion without the need for TNFα. Taken together these studies indicate that, in addition to other inflammatory mediators and acute phase proteins, cytokines and chemokines are produced by hepatocytes, which may participate in hepatotoxic responses. The events responsible for their expression involve cellular redox changes.

Key Words: **hepatotoxicity; interleukin-8; chemokines; vanadium pentoxide; cadmium; Hep G2; hepatocytes; reactive oxygen species; oxidative stress.**

Liver injury, in response to a variety of hepatotoxic chemicals and infectious agents including heavy metals, alcohol, carbon tetrachloride, acetaminophen, and viral hepatitis, is often associated with a chronic inflammatory response involving cytokines, particularly tumor necrosis factor-α (TNFα) and interleukin (IL)-8 (Blazka *et al.*, 1995; Kayama *et al.*, 1995; Schook *et al.*, 1992; Maltby *et al.*, 1996; Bruccoleri *et al.*, 1997; Iimuro *et al.*, 1997). Pathophysiological responses proximally or distally mediated by TNFα in the liver include inflammatory cell infiltration, hyperlipidemia, oxygen radical generation, fibrogenesis, and cholestasis (Andus *et al.*, 1991; Dinarello, 1993; Feingold and Grufeld, 1987). TNFα, at low concentrations, also plays a protective role in the liver as it can activate acute phase responses, induce hepatocyte proliferation, and stimulate manganese superoxide dismutase production (Andus *et al.*, 1991; Beyer and Theologides, 1993; Wong and Goeddel, 1988). Neutrophil chemoattractant cytokines from the α-chemokine superfamily, characterized by two cysteines separated by a single amino acid (CXC), such as IL-8 in humans, macrophage inflammatory protein (MIP)-2 in mice, and cytokine-induced neutrophil chemoattractant (CINC) in rats, represent one of the most stable and potent families of neutrophil chemotactic and activating factors (Baggiolini *et al.*, 1994; Ben-Barach *et al.*, 1995). Chemokine-mediated influx and activation of neutrophils and their subsequent release of reactive oxygen species (ROS) in response to focal sites of organ damage are believed to participate in liver damage following exposure to many hepatotoxic agents (Ganey and Schultze, 1995; Laskin, 1990).

Studies on hepatic cytokine expression have focused primarily on Kupffer cells, the resident liver macrophages, which are considered as their primary source. In addition, endothelial cells, which represent about 15% of the total liver cell population, as well as Ito cells, respond to various stimuli by secreting proinflammatory cytokines (Laskin, 1990). In contrast to nonparenchymal cells, very little information is available on the role of hepatocytes in cytokine production despite the fact that they compose the vast majority of cells in the liver. We previously demonstrated that the nuclear transcription factor (NF)-κB family of transcription factors is rapidly activated in hepatocytes in direct response to hepatotoxic agents, such as

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carbon tetrachloride and acetaminophen (Blazka *et al.*, 1995, 1996; Bruccoleri *et al.*, 1997). As the NF-κB family is involved in the transcriptional regulation of TNFα and IL-8 (Oliveria *et al.*, 1994; Banerjee *et al.*, 1989) and can be activated by ROS (Schreck *et al.*, 1992), we hypothesized that hepatocytes are capable of producing inflammatory cytokines in response to hepatotoxic agents, many of which are metals with pro-oxidant activity. The metals selected as models for study were cadmium chloride ($CdCl_2$) and vanadium pentoxide (V_2O_5), both of which can readily produce oxidative damage (Dargel, 1992; Manca *et al.*, 1991; Wenning and Kirsch, 1988).

MATERIALS AND METHODS

Animals. Adult Fisher 344 rats and C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). TNFα double receptor (p55/p75) knockouts (C57BL/6x129) were kindly provided by Immunex Corp. (Seattle, WA) through Dr. Larry Schook (University of Minnesota, St. Paul, MN). All transgenic mice were rederived (Charles River) and maintained under specific pathogen-free conditions in AALAC certified facilities at NIOSH.

Chemicals. Hydrogen peroxide (H_2O_2), $CdCl_2$, and V_2O_5 were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human TNFα and antibodies to recombinant TNFα were purchased from R&D Systems (Minneapolis, MN). 1,1,3,3,-Tetramethyl-2-thiourea (TMTU; Sigma) or *N*-acetyl-l-cysteine (NAC; Sigma) was dissolved and diluted in RPMI culture medium to the desired concentration immediately prior to use. The metals were suspended in culture medium and were sonicated immediately before addition to the cell cultures.

Cell culture and treatment. Hepatocytes were isolated from 2- to 3-month old male mice or rats and prepared by a modification of the procedure of Seglen (1976). Briefly, rodents were anesthetized with 100 mg/kg phenobarbital. The liver was exposed and perfused *in situ* through the portal vein at a flow rate of 20 ml/min with 200 ml of warmed (37°C) perfusion buffer (212 mM NaCl, 6 mM KCl, 0.6 mM $MgSO_4$, 0.7 mM KH_2PO_4, 25 mM $NaHCO_3$, and 5 mM glucose; pH 7.5) containing 0.5 mM EDTA. Flow was switched without interruption to 200 ml of perfusion buffer containing 0.035% Type IV collagenase (Sigma) and 1 mM $CaCl_2$. The liver was removed and placed into a petri dish containing approximately 25 ml of Williams E medium (Sigma). The capsule was ruptured and the cells were mechanically dispersed. The suspension was filtered through two layers of sterile gauze and the hepatocytes were further enriched by an isodensity Percoll (Pharmacia Fine Chemicals Inc., Piscataway, NJ) centrifugation method (Kreamer *et al.*, 1986). Viability, assessed by Trypan blue exclusion, was always greater than 90%. Aliquots of 1×10^6 cells in 2 ml volumes were seeded into six-well culture dishes in RPMI 1640 culture media (GIBCO, BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 2 mM L-glutamine. The wells of the culture dishes were preconditioned by treating with 0.5 ml of 0.25% Type VII rat tail collagen (Sigma) in 0.1% acetic acid, air-dried overnight in an operating laminar flow hood, washed with cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), and equilibrated with culture medium for 4 h. Following incubation at 37°C for 1 h to allow for adherence, the cell cultures were treated with media in which the test agents had been dissolved. The cells were collected following 2 h of incubation for RNA isolation or supernatants were collected following 18 h of incubation for quantitation of cytokine secretion as preliminary studies indicated that these represent optimal time points for sampling.

Hep G2 cells (ATCC, Rockville, MD), a human hepatocellular carcinoma cell line, were grown and collected under standard conditions (Giri *et al.*, 1992). Cells were collected, centrifuged, and suspended to a concentration of 1×10^6 cells/ml in culture medium and cell viability was assessed by Trypan blue exclusion (always 98%). The cells were seeded in 1 ml volumes into 24-well culture dishes for supernatant collection or 3 ml volumes into 12-well culture dishes for RNA extraction. Following incubation at 37°C for 1 h to allow for cell adherence, the cultures were treated with test agents and cells were collected following 2 h of incubation for RNA extraction or supernatants were collected following 18 h of incubation to determine the concentration of secreted cytokines. In preliminary experiments these time points were shown to be optimal for cytokine mRNA expression and secretion, respectively (data not shown).

Semiquantitative reverse transcriptase–polymerase chain reaction (RT–PCR). Cells were collected and homogenized in 1 ml of Ultraspec RNA (Biotecx Laboratories, Houston, TX) and total cellular RNA was extracted according to the manufacturer's procedure. cDNA was synthesized as described previously (Simeonova and Luster, 1996) and the reaction mixture was diluted with distilled water to 100 μl. PCR primers for human G3PDH, IL-8, and TNF-α were purchased from Clontech (Palo Alto, CA). Five-microliter aliquots of the synthesized cDNA were added to 45 μl of PCR mixture containing 5 μl of 10× PCR buffer, 1 μl deoxynucleotides (1 mM each), 0.5 μl of sense and antisense primers (0.15 μM), and 0.25 μl DNA polymerase (GeneAmp PCR kit, Perkin Elmer). The reaction mixture was covered with an AmpliGem wax tablet (Perkin Elmer) and amplification was initiated by 1 min of denaturation at 95°C for 1 cycle, followed by 25 to 35 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s using a GeneAmp PCR System 9600 DNA Thermal Cycler (Perkin Elmer Cetus). After the last cycle of amplification, the samples were incubated at 72°C for 7 min. For each set of primers, dilutions of cDNA were amplified for 20, 23, 25, 28, 30, and 35 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength (Kayama *et al.*, 1995). If necessary, the concentrations of cDNA were readjusted (normalized to G3PDH) and the PCR was repeated. The specificity of the PCR bands were previously confirmed by restriction site analysis of the amplified cDNA, which generated restriction fragments of the expected size (data not shown).

Amplified PCR products were separated electrophoretically on 1% agarose gel (UltraPure, Sigma) at 75 V for 60 min and visualized by UV illumination after staining with 0.5 μg/ml ethidium bromide. The molecular weight marker ΦX174 DNA *Hae*III digest was purchased from Sigma. Gels were photographed with Type 55 positive/negative film (Polaroid, Cambridge, MA) and scanned with a computerized laser densitometer.

Cytokine secretion. TNFα activity was measured in culture supernatants using the L929 mouse fibroblast (ATCC) lysis bioassay in the presence of 6 μg/ml actinomycin D (Matthews and Neale, 1987). A standard curve was prepared using mouse recombinant TNFα (R&D Systems). Cytolysis was calculated from the reduction in mean absorbency at 570 nm in a microplate reader. For IL-8 quantitation, 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with mouse anti-human IL-8 monoclonal antibody (R&D Systems). Nonspecific binding sites were blocked by treating the plates with 1% BSA in PBS for 2 h. IL-8 standards or test samples were added for 2 h followed by goat anti-human IL-8 IgG (R&D Systems) antibody for 2 h and then a detection antibody (swine anti-goat IgG, peroxidase conjugated) at a 1:7500 dilution for 1 h at room temperature. All samples were added in 100-μl aliquots and the plates were washed with PBS between each step. *O*-Phenylenediamine dihydrochloride substrate (0.4 mg/ml) in phosphate–citrate buffer supplemented with 4.4 mM H_2O_2 was added for 10 min at room temperature and the enzymatic reaction was stopped by the addition of 50 μl of 4 N HCl. The plates were read at 490 nm and mean absorbency was analyzed using a curve-fitting program. MIP-2 secretion was determined using a commercial system (Biosource, Camarillo, CA). CINC was quantitated by an ELISA procedure. Briefly, microtiter plates (Dynatech Laboratories) were coated overnight at 4°C with polyclonal rabbit anti-rat CINC antibody (Pepro Tech Inc., Rocky Hill, NJ) at a concentration of 2 μg/ml. Nonspecific binding sites were blocked by treating the plates with 1% BSA in PBS for 2 hrs. Recombinant rat CINC (Pepro Tech Inc.) or test samples were added for 2 h at 37°C followed by 1 h of incubation with polyclonal anti-rat CINC antibody (Pepro Tech Inc.), which had been labeled with biotin using a commercial kit (Pierce, Rockford, IL). The bound biotin was detected follow-

ing incubation with streptavidin peroxidase (Pierce). *O*-Phenylenediamine dihydrochloride substrate (0.4 mg/ml) in phosphate–citrate buffer supplemented with 4.4 mM H_2O_2 was added for 15 min at room temperature and the enzymatic reaction was stopped by the addition of 50 μl of 4 N HCl. The plates were read at 490 nm and mean absorbency was analyzed using a curve-fitting program.

TNF neutralization. Hep G2 cells were allowed to adhere and the culture media was replaced with fresh media containing 250 μl of recombinant human anti-TNF antibodies (Genzyme). After incubation at 37°C for 1 h, the cells were treated with either 10 μM $CdCl_2$, 1.0 μM V_2O_5, or 10 ng/ml recombinant TNFα and supernatants were collected for IL-8 quantitation following an additional 18 h of incubation.

Chemotaxis. Human peripheral blood neutrophils were isolated using Ficoll-Hypaque (LSM, Organon Teknika Corp., Durham, NC) followed by erythrocyte sedimentation in dextran T500 (Pharmacia) and removal of contaminating erythrocytes by hypotonic lysis. The neutrophils were adjusted to 2.5×10^6 cells/ml in RPMI 1640 culture media. Test samples were diluted in an equal volume of media and added in duplicate to the bottom wells of chemotaxis chambers (Neuro Probe Inc., Cabin John, MD). The neutrophil suspension (250 μl) was added to the top chamber and separated from the samples by a 3-μm polycarbonate filter (PVP-free, Poretics Corp., Livermore, CA). The positive control consisted of 10^{-7} M FMLP (Sigma) and the negative control was HBSS. For neutralizing studies, the test samples were incubated with a 1:100 dilution of polyclonal anti-human IL-8 antiserum (R&D Systems) for 45 min at 37°C before adding to the chamber. Following incubation of the chamber assemblies at 37°C for 1 h, the filters were removed and stained by hematoxylin–eosin. Neutrophils that migrated to the bottom of the chamber were enumerated by using a 400× magnification (10 fields). Chemotactic activity is presented as the percentage of the positive control (i.e., 10^{-7} M FMLP).

Lactate dehydrogenase (LDH) estimation. LDH, a measure of cytoplasmic leakage, was determined in culture supernatant using a single reagent system (Sigma) and measured at 340 nm (Simeonova and Luster, 1996).

Statistical analysis. The Student's *t* and Dunnett's tests were used in single and multiple comparisons of means with a common control group, respectively (Statview, Abacus Concepts, Berkeley, CA). Statistically significant differences were reported at $p < 0.05$.

RESULTS

To establish whether hepatocytes produced chemokines in response to metals, either Hep G2 cells or freshly prepared rodent hepatocytes were cultured for 18 h in the presence of $CdCl_2$ or V_2O_5 and IL-8, MIP-2, or CINC levels were determined in the supernatants. Freshly prepared rat and mouse hepatocytes readily secreted CINC and MIP-2, respectively, in response to $CdCl_2$, while Hep G2 cells secreted IL-8 (Fig. 1). The dose–response curves for all three chemokines were similar except in mouse hepatocyte cultures where a decrease in MIP-2 secretion was observed at the highest concentration of $CdCl_2$ tested. This was apparently due to cytotoxicity and was the only concentration of cadmium and cell type where cytotoxicity was observed, the latter indicated by a 35% increase in LDH leakage (data not shown). Incubation of V_2O_5 with hepatocytes also induced chemokine secretion (Fig. 2). However, peak chemokine secretion occurred at slightly lower concentrations than observed with $CdCl_2$. As with $CdCl_2$, mouse hepatocytes were more sensitive to the cytotoxic effects of V_2O_5 than hepatocytes from other species as indicated by LDH

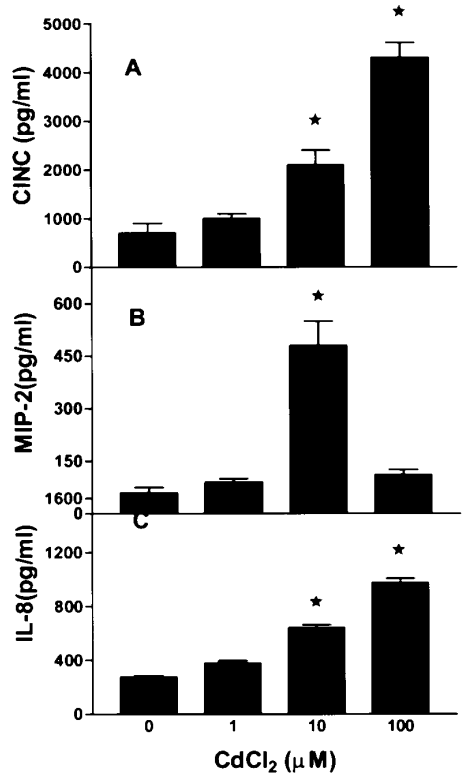

FIG. 1. Chemokine secretion from hepatocytes in response to $CdCl_2$. Freshly isolated rat hepatocytes (A), mouse hepatocytes (B), or Hep G2 cells (C) were incubated for 18 h with concentrations of CdCl, and supernatants were collected for CINC, MIP-2, or IL-8 determination, respectively, as described in Materials and Methods. Values represent means ± SE of four individual samples. Results are representative of at least three replicate experiments. *Significantly different from control at $p < 0.05$.

leakage (data not shown). To determine whether the chemokine activities observed in culture supernatants were reflected at the transcriptional level, RNA was isolated from Hep G2 cells after 2 h of incubation with $CdCl_2$ or V_2O_5 and the relative numbers of IL-8 mRNA transcripts were determined by RT–PCR (Fig. 3). RNA concentrations among test samples were normalized using the constitutively expressed G3PDH gene. Relative increases in mRNA for IL-8 were observed in cells following incubation with metals at concentrations that were consistent with secreted levels.

Neutrophil chemotactic activity in hepatocyte supernatants was examined following incubation with the metals. As shown in Fig. 4, culture supernatants from metal-exposed Hep G2 cells induced a vigorous chemotactic response that was inhibited by over 60% when the supernatants were incubated with antibodies to IL-8. Thus, the majority of the chemotactic activity secreted can be assumed to represent biologically active IL-8.

As previous studies demonstrated that ROS can induce TNFα secretion in hepatocytes, we examined whether $CdCl_2$ or V_2O_5 were also capable of stimulating TNFα secretion. As shown in Fig. 5, bioactive TNFα was observed in culture

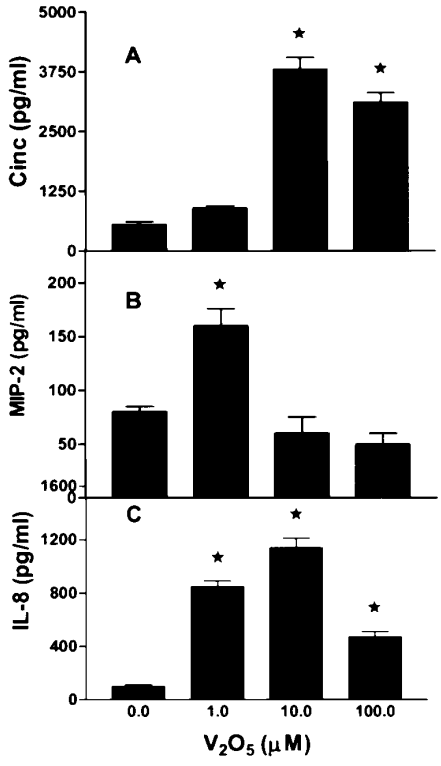

FIG. 2. Chemokine secretion from hepatocytes in response to V_2O_5. Freshly isolated rat hepatocytes (A), mouse hepatocytes (B), or Hep G2 cells (C) were incubated for 18 h with concentrations of V_2O_5, and supernatants were collected for CINC, MIP-2, or IL-8 determination, respectively. Values represent means ± SE of four individual samples. Results are representative of three replicate experiments. *Significantly different from controls at $p < 0.05$.

supernatants following overnight incubation with either of the test metals. However, compared to V_2O_5, relatively high concentrations of $CdCl_2$ that were cytotoxic (data not shown) were needed to stimulate TNFα secretion.

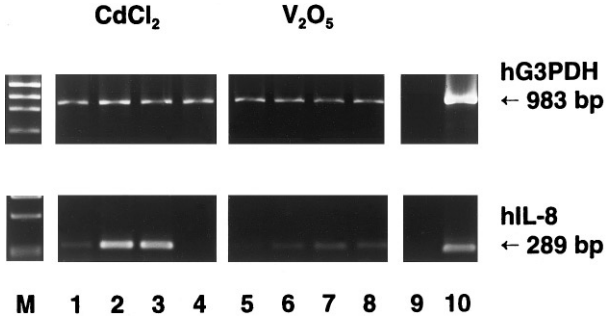

FIG. 3. IL-8 gene expression in Hep G2 cells treated with metals. Hep G2 cells (3×10^6 cells/well) were treated for 2 h with $CdCl_2$ or V_2O_5, and total RNA was isolated and prepared for RT–PCR as described in Materials and Methods. Lane M, molecular weight marker (ΦX174 DNA *Hae*III digest); lanes 1–4, 0, 10, 100, and 1000 μM $CdCl_2$; lanes 5–8, 0, 0.5, 2.5, and 10 μM V_2O_5; lane 9, negative (water); and lane 10, commercial positive control. Loading equivalence was verified by equal intensities of G3PDH mRNA. The expected sizes of the PCR products were 983 base pairs (bp) for hG3PDH and 289 bp for IL-8.

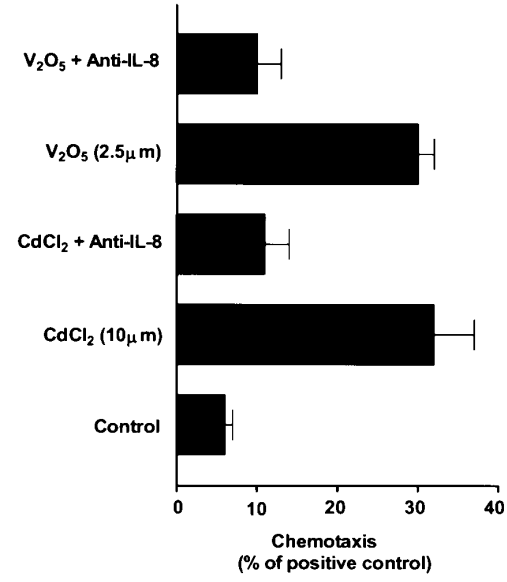

FIG. 4. Chemotactic activity in Hep G2 cell supernatants. Supernatants from Hep G2 cells were treated for 18 h with $CdCl_2$ or V_2O_5 and ±30 min of incubation with a 1:100 dilution of anti-IL-8 antiserum. A mean of 285 neutrophils per high power field was observed and values presented as a percent of the positive control (10^{-7} M FMLP). Values shown are representative of duplicate experiments.

Since hepatocytes under certain circumstances secrete TNFα, and TNFα, in turn, can stimulate chemokine secretion, it was necessary to establish whether the chemokine response induced by the metals was mediated via TNFα. Thus, experiments were conducted in which Hep G2 cells were treated with $CdCl_2$ or V_2O_5 in the presence of neutralizing TNFα antibodies (Fig. 6). The addition of TNFα antibodies prevented IL-8 secretion in the presence of added TNFα (positive control). However, IL-8 secretion induced by either of the metals was not inhibited significantly by neutralizing antibodies, indicating TNFα was not involved in the IL-8 response. Studies were

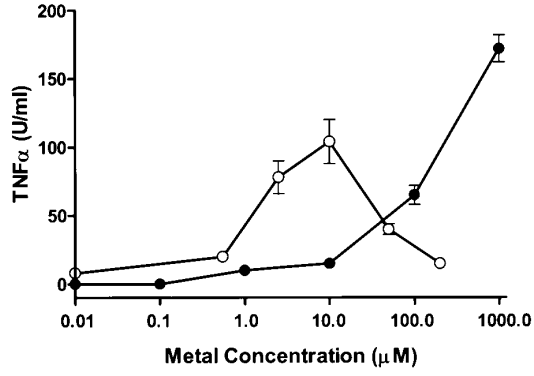

FIG. 5. TNFα secretion in Hep G2 cell cultures treated with metals. Hep G2 cells were incubated in the presence of $CdCl_2$ (●) or V_2O_5 (○) for 18 h and TNFα concentrations measured in the supernatants. Each value represents the mean ± SE of triplicate samples.

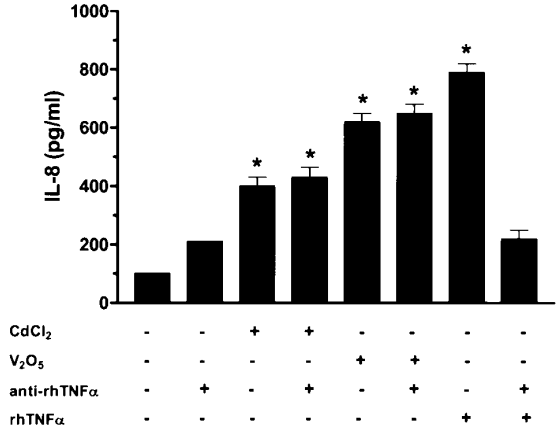

FIG. 6. IL-8 secretion in the presence of neutralizing antibodies to TNFα. Samples containing anti-TNFα antibodies contained 250 neutralizing U/ml. $CdCl_2$ (10 μM), V_2O_5 (10 μM), or TNFα (10U/ml) were added to Hep G2 cell cultures 30 min after the antibody and IL-8 was measured in the supernatants after 18 h of incubation. Values represent means ± SE of triplicate cultures. *$p < 0.05$ vs controls.

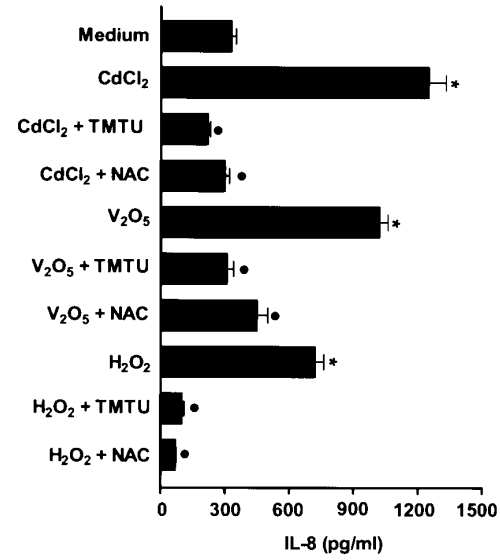

FIG. 8. Inhibition of IL-8 secretion by antioxidants. Hep G2 cells (1×10^6 cells/well) were incubated for 18 h in the presence of 10 mM TMTU or 10 mM NAC and either 10 μM $CdCl_2$, 2.5 μM V_2O_5, or 0.25 mM H_2O_2. IL-8 concentrations in the supernatants were measured as described in Materials and Methods. Values represent means ± SE of four individual samples. *Significantly different ($p < 0.05$) from control cells. ●Significantly different ($p < 0.05$) from stimulated cells. Results are representative of three replicate experiments.

also conducted using transgenic mice that do not express TNF receptor 1 or 2. As shown in Fig. 7, MIP-2 secretion induced by lipopolysaccharide (LPS) (negative control), V_2O_5 or $CdCl_2$ was similar in hepatocytes from wild-type and knockout mice while MIP-2 secretion was suppressed in hepatocytes from the knockout mice when stimulated with TNFα. This further confirms that metal-induced MIP-2 expression in hepatocytes is independent of TNFα secretion.

Hepatotoxicity caused by exposure to toxic agents is often associated with the pro-oxidant activity of the agent. Furthermore, the liver, as the primary organ involved in redox cycling and the generation of oxidative enzymes, is responsible for considerable oxidant activities. Since ROS can mediate cell signaling events leading to increased expression of chemokines, the role of oxidative stress in IL-8 and MIP-2 synthesis was examined. The addition of H_2O_2 to hepatocytes induced IL-8 secretion and this was inhibited by the addition of various antioxidants including TMTU and NAC (Fig. 8). IL-8 secretion by $CdCl_2$ and V_2O_5 in hepatocytes was also prevented when the cells were incubated in the presence of TMTU or NAC. Similar results were obtained when MIP-2 secretion was examined in freshly isolated mouse hepatocytes (Fig. 9). The addition of these antioxidants to Hep G2 cells or hepatocytes, alone, had no effect on constitutive chemokine secretion or cytotoxicity (data not shown).

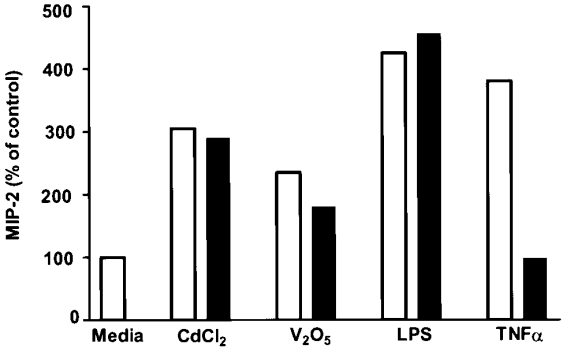

FIG. 7. MIP-2 secretion in hepatocytes from TNF receptor knockout and wild-type mice. Freshly isolated hepatocytes from wild-type (open) or transgenic (closed) litter mates were cultured for 18 h in the presence of 10 μM $CdCl_2$, 1 μM V_2O_5, 1 μg/ml LPS, or 10 U/ml TNFα and MIP-2 was measured in the supernatants. Constitutive MIP-2 expression in cell cultures from hepatocytes isolated from wild-type mice was 20 pg/ml and from knockouts was 15 pg/ml. Values are presented as percent from control and are representative of three replicate experiments.

DISCUSSION

The present studies provide evidence that hepatocytes are capable of producing cytokines and chemokines in response to specific hepatotoxins, including $CdCl_2$ and V_2O_5. In-depth studies with the CXC family of chemokines, including IL-8, MIP-2, and CINC suggested that this response occurs in both human and rodent species, is mediated by the generation of ROS, and is independent of TNFα production. This confirms and extends previous studies (W. Dong *et al.*, unpublished observations; Thornton *et al.*, 1990), which demonstrated that IL-8 is secreted by hepatocytes in response to common simulators such as LPS, phorbol ester, and TNFα. The ability of H_2O_2 to directly stimulate chemokine secretion is consistent

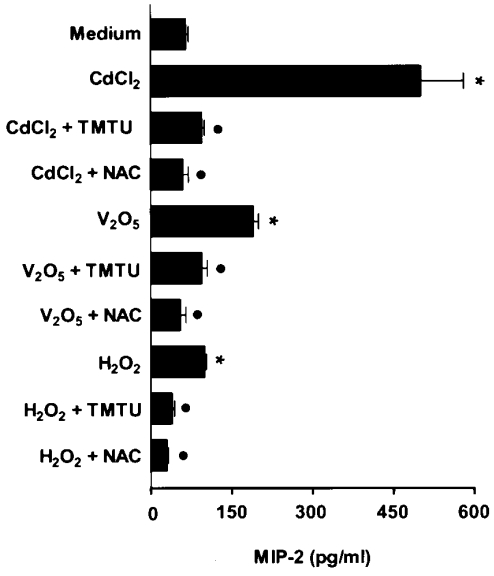

FIG. 9. Inhibition of MIP-2 expression by antioxidants. Mouse hepatocytes (1×10^6 cells/well) were incubated for 18 h in the presence of 10 mM TMTU or 10 mM NAC and either 10 μM $CdCl_2$, 2.5 μM V_2O_5, or 0.25μM H_2O_2. MIP-2 concentrations in supernatants were measured as described in Materials and Methods. Values represent means ± SE of four individual samples. *Significantly different ($p < 0.05$) from control cells. ●Significantly different ($p < 0.05$) from stimulated cells. Results are representative of three replicate experiments.

with observations demonstrating that ROS regulate the expression of a variety of genes involved in inflammatory and stress responses through activation of nuclear transcription factors (Schreck *et al.,* 1992; Remick and Villareti, 1996). In this respect, we have recently shown that H_2O_2 activates NF-κB and NF-IL-6, resulting in their binding to regulatory elements in the IL-8 promoter and production of IL-8 protein in lung epithelial cells (Simeonova and Luster, 1996; Simeonova *et al.,* 1997). The exact mechanisms by which H_2O_2 activates transcription factors have not been defined, although protein kinase C- and phosphorylation-dependent events have been implicated.

Although $CdCl_2$ has been studied most extensively (Lauwerys, 1979), hepatotoxicity occurs following exposure to many heavy metals, including vanadium (Wenning and Kirsch, 1988). Free radicals generated by hepatic metabolism or by the hepatotoxicant itself are thought to produce cell damage via oxidation of unsaturated fatty acids of phospholipids (Comporti, 1985). Lipid peroxidation is a feature of hepatocyte injury by toxic agents including heavy metals (Dargel, 1992; Seig and Billings, 1997; Ohsawa, 1997). In addition to hepatotoxic chemicals with direct oxidant activities, ROS within the liver can originate from endogenous sources such as by-products of activated Kupffer cells, oxidant enzyme activities, and altered cellular redox states following mitochondrial damage (Fridovich, 1983; Aust *et al.,* 1993). Cadmium is thought to damage endothelial cells of the hepatic sinusoids and hepatocyte destruction is secondary, resulting from localized ischemia caused by the destruction of the hepatic microcirculation (Nolan and Shaikh, 1986). This is supported by observations of cadmium-induced histopathological changes in both parenchymal and vascular endothelium (McKim *et al.,* 1992) as well as *in vitro* studies using hepatic endothelial cells (Liu *et al.,* 1992). Thus, hepatotoxicity by heavy metals is a complex series of events involving multiple cell types and genetic as well as epigenetic events with ROS playing a pivotal role.

Hepatic cytokines and chemokines are involved in both pathological and repair processes in the liver. For example, the importance of TNFα as a regulator of hepatic repair was recently demonstrated in rats in which administration of TNFα antibodies prior to partial hepatectomy (Akerman *et al.,* 1992) or induction of hepatotoxicity by carbon tetrachloride (Bruccoleri *et al.,* 1997) prevented normal regenerative processes. This is consistent with the reported ability of TNFα to serve as a hepatocyte mitogen (Beyer and Theologides, 1993). On the other hand, locally produced TNFα and IL-8 have been reported to be proximal mediators of several forms of liver injury (Andus *et al.,* 1991; Dinarello, 1993; Feingold and Grufeld, 1987). The association between TNFα and IL-8 and prooxidant activities in the liver may be central to the effects of many classical hepatotoxic agents and conditions, including acetaminophen, carbon tetrachloride, and heavy metals as well as ischemia and alcohol-induced cirrhosis (Blazka *et al.,* 1995; Kayama *et al.,* 1995; Schook *et al.,* 1992; Bruccoleri *et al.,* 1997; Armendariz-Borunda *et al.,* 1991; Maltby *et al.,* 1996). The hypothesis that links these observations can be stated as follows: initial liver injury via chemical hepatotoxicants or ischemia results in lipid peroxidation and focal areas of hepatic damage. As a consequence of this damage, local Kupffer cells and monocytes are activated and secrete inflammatory mediators, including cytokines and chemokines. Chemokines, such as IL-8 in humans or MIP-2 and CINC in rodents, function as neutrophil chemoattractants and activators and it is the release of ROS from these infiltrating neutrophils that is responsible for general liver damage. This hypothesis is evidenced, in part, by the ability to attenuate hepatotoxicity by either Kupffer cell depletion, neutrophil depletion, antioxidant treatment, or cytokine neutralization (Ganey and Schultze, 1995; Laskin, 1990; Blazka *et al.,* 1995).

Although antioxidants effectively inhibited chemokine secretion induced by either metal, V_2O_5 was more effective than $CdCl_2$ in inducing TNFα and chemokine secretion. The difference between the two metals may be associated with their ability to serve as oxidants, as V_2O_5 would likely be more reactive. Both the membrane-permeable thiol-containing scavengers, NAC and TMTU, were effective inhibitors. Thiol-containing antioxidants are effective scavengers of OH · radicals, which maintain the intracellular concentrations of glutathione, thus, generally increasing the ability of cells to scavenge ROS. Radicals produced by hydrogen atom abstraction from thiol-containing antioxidants might themselves exert

deleterious biological effects and may account for the slightly reduced effectiveness of NAC, compared to TMTU, to inhibit metal- or H_2O_2-induced IL-8 secretion.

In summary, increasing evidence has indicated that a cytokine network exists in the liver that is involved, not only in physiological responses, but also, under certain circumstances, pathological and repair processes following hepatic injury. While nonparenchymal cells, particularly Kupffer cells, represent a major source of hepatic cytokines, the present studies demonstrate that parenchymal cells not only respond to cytokines but also secrete specific cytokines in response to chemical injury. Although the role cytokines play in liver damage and repair is increasingly being appreciated, the exact role hepatocyte-derived cytokines play in these processes is unknown. It is possible that they act only at local sites of injury to help facilitate repair. Alternatively, these cytokines may not be confined to the liver, and their release from hepatocytes and presence in hepatic venous circulation may contribute to extrahepatic disease processes (Thornton *et al.*, 1990).

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