Cytokine Response and Oxidative Stress Produced by Ethanol, Acetaldehyde and Endotoxin Treatment in HepG2 Cells

Ma. C. Gutierrez-Ruiz PhD[1], Luis E. Gomez Quiroz MSc[1], Elizabeth Hernandez MSc[1], Leticia Bucio PhD[1], Veronica Souza MSc[1], Luis Llorente PhD[2] and David Kershenobich PhD[3]

[1]Department of Health Sciences, Metropolitan Autonomous University, and Departments of [2]Immunology, and [3]Gastroenterology, National Institute of Medical Sciences and Nutrition Salvador Zubiran, Mexico City, Mexico

Key words: HepG-2 cells, cytokines, antioxidant enzymes, ethanol, acetaldehyde, lipopolysaccharide

Abstract

Background: Inflammatory mediators, including cytokines and reactive oxygen species, are associated with the pathology of chronic liver disease. Hepatocytes are generally considered as targets but not producers of these important mediators.

Objectives: To investigate whether cells of hepatocellular lineage are a potential source of various cytokines we estimated the expression and secretion of tumor necrosis factor alpha, transforming growth factor beta1, and interleukins 1beta, 6 and 8 in the culture of well-differentiated human HepG2 cells treated for 24 hours with ethanol, acetaldehyde and lipopolysaccharide. Lipid peroxidation damage, glutathione content and glutathione peroxidase, catalase and superoxide dismutase activity were also determined.

Methods: HepG2 cells were treated for 24 hours with ethanol (50 mM), acetaldehyde (175 μM) and LPS (1 μg/ml). TNF-α, TGF-β, IL-1β, IL-6 and IL-8 mRNA were determined by reverse transcriptase polymerase chain reaction and secretion by enzyme-linked immunoassay. Lipid peroxidation damage, glutathione content and antioxidant enzyme activities were determined spectrophotometrically.

Results: Exposure to ethanol for 24 hours induced the expression of TNF-α and TGF-β_1, secretion of IL-1β and TGF-β_1 and decreased catalase activity. Acetaldehyde markedly increased TNF-α and IL-8 expression, stimulated IL-1β and IL-8 secretion, increased lipid peroxidation damage and decreased catalase activity, while LPS exposure induced the expression of TNF-α, TGF-β_1, IL-6 and IL-8, the secretion of TGF-β_1, IL-1β, IL-6 and IL-8, and a decrease in catalase activity. No change in GSH, GSHPx or SOD was found in any experimental condition.

Conclusions: The present studies confirm and extend the notion that hepatocytes respond to ethanol, acetaldehyde and LPS-producing cytokines. Oxidative stress produced by the toxic injury plays an important role in this response through up-regulation of inflammatory cytokines.

IMAJ 2001;3:131–136

Cytokines and growth factors are the major orchestrators of host defense processes and, as such, are involved in responses to exogenous and endogenous insults, repairs and restoration of homeostasis. Because of its anatomical location and role in drug and xenobiotic detoxification, the healthy liver is confronted almost continuously with factors that induce injury, such as pro-inflammatory cytokines and reactive oxygen species. TNF-α induction is known to be one of the earliest events in hepatic inflammation, triggering a cascade of other cytokines that cooperate to kill hepatocytes, recruit inflammatory cells, and initiate a wound-healing response that includes fibrogenesis. Ethanol and its metabolites, mainly acetaldehyde, as well as endotoxins (LPS) have been observed to contribute directly to an inflammatory response mediated by cytokines and growth factors such as IL-1β and TNF-α [1]. In this regard, it is important to acknowledge the growing evidence that TNF-α is required for ethanol-induced liver damage [2].

ROS has been implicated in the pathogenesis of many forms of liver disease. When liver cells are exposed to excess ROS, oxidative stress occurs and affects many cellular functions. This can alter gene expression through activation and transcription factors, such as nuclear factor-κB and activating protein 1, leading to up-regulation of cytokines, chemokines, adhesion molecules, survival genes, etc. Although much has been published concerning lipid peroxidation, which is no doubt a useful marker of oxidative stress, the role of this process in cell damage remains unclear. Many enzymes and co-factors may be affected by oxidative stress. These may include glutathione, glutathione-related enzymes, as well as antioxidant enzymes. Some physiologically important antioxidant enzymes include glutathione peroxidase, catalase and superoxide dismutase as well as other reductants such as glutathione. These enzymes and low molecular weight substances are important in protecting against ROS such as superoxide and hydrogen peroxide, which are produced in response to oxidative stress.

Although cytokines are known to be involved in the regulation of a variety of hepatocellular functions, hepatocytes themselves are generally considered as merely targets and not major producers of these important mediators. In order to investigate whether cells of hepatocellular lineage are a potential

LPS = lipopolysaccharide
TNF-α = tumor necrosis factor alpha
TGF-β_1 = transforming growth factor beta1
IL = interleukin
GSH = glutathione
GSHPx = glutathione peroxidase
SOD = superoxide dismutase

ROS = reactive oxygen species

source of various cytokines we estimated the expression and secretion of TNF-α, TGF-β_1, IL-1β, IL-6 and IL-8 in the culture of well-differentiated human HepG2 cells treated for 24 hours with ethanol, acetaldehyde and LPS. Lipid peroxidation damage, GSH content and GSHPx, catalase and SOD activity were also determined.

Methods

Cell culture

The human hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (Maryland, USA) at passage number 79. All cells used in this work were between passage 90 and 120. HepG2 cells were routinely grown in monolayer culture in Williams E medium supplemented with 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA), penicillin (100 units/ml) and streptomycin (100 mg/ml). Cells were grown at 37°C in disposable plastic bottles (Nunc, USA) in a humidified atmosphere of 5% CO_2/95% air. The medium was replaced twice a week, and cells were trypsinized and diluted every 7 days at a ratio of 1:3.

Experimental design

Lipopolysaccharide from *Salmonella typhimurium* (Sigma, St. Louis, MO, USA), ethanol, and acetaldehyde (Merck) were used. Reagent grade 95% ethanol was redistilled prior to addition to the culture medium. Stock solution of ethanol and acetaldehyde were prepared daily. HepG2 cells were incubated with ethanol (50 mM) and acetaldehyde (175 μM) in serum-free medium, previously pre-equilibrated with 5% CO_2/95% air, for up to 24 hours, and the flasks were kept tightly close and wrapped in parafilm to prevent evaporation of ethanol and acetaldehyde. In the case of multichambers, these were sealed with plastic tape. HepG2 cells were seeded at a density of 2.5 x 10^4 cells/cm^2. All cells were seeded at the same time. The media were changed every 24 hours.

Treatments

Twenty-four hours after seeding cells, culture media were changed for one containing 50 mM ethanol, 175 μM acetaldehyde (initial concentrations) or 1 μg/ml LPS. After 24 hours, the medium was collected and centrifuged for 15 min at 4,000 rpm and stored at -80°C until assays were performed. Cells were washed twice with phosphate buffer, scraped from bottles to determine enzyme activities, GSH content and lipid peroxidation, and to obtain cytokine and growth factors mRNA. Control cells were seeded at the same time as treated cells. They were maintained under the same conditions but without the addition of ethanol, acetaldehyde or LPS.

Neutral red assay

This assay was done as described by Borenfreund and Puerner [3], and the incorporation of the supravital dye neutral red into lysosomes of viable cells was determined. The amount of dye, after extraction from the lysosomes, was quantified spectrophotometrically and compared with that of the control cells.

Lipid peroxidation

Lipid peroxidation was assayed by determining the rate of production of thiobarbituric acid-reactive components [4]. Cells ($3.6x10^6$) grown on a 80 cm^2 flask were washed with isotonic NaCl solution, scraped and resuspended in 1 ml PBS. An aliquot of 0.9 ml was used for measured lipid peroxidation and protein was assayed with the remaining 0.1 ml. Two ml of a solution containing 15% trichloroacetic acid, 0.25 N HCl and 0.5% thiobarbituric acid were added to the suspension. Samples were heated for 25 minutes in a boiling water bath, cooled and centrifuged for 10 min at 4,000 rpm. The absorbance of the supernatant fraction was determined at a wavelength of 535 nm. An extinction coefficient of $1.56x10^5$ $M^{-1}cm^{-1}$ was used to calculate the concentration of malondialdehyde. Values were expressed as pmol of MDA per mg protein.

GSH content assay

Cells were washed twice with PBS and gently scraped into 0.6 ml PBS. An aliquot was taken to determine protein. Reduced glutathione was measured by the method of Tietze [5]. Standard curve was established using known GSH dilutions.

Enzyme activities

Cells ($3.5x10^6$ cells/cm^2) were seeded and treated as mentioned in the experimental conditions. Cells were washed with PBS and scraped with a rubber policeman. Cell suspensions were then lysated by sonication. An aliquot of 50 μl was taken for protein determination and the rest of the volume centrifuged during 45 min at 48,000 rpm at 4°C. Supernatant was used for enzyme activity determinations.

Superoxide dismutase

SOD was determined based on its ability to inhibit the reduction of nitro-blue tetrazolium by superoxide. A reaction system of 3 ml contained 0.1 M EDTA, 1.5 mM NBT, 0.067 M PBS pH 7.8 was incubated for 10 min with a series of samples ranging from 5 to 300 μl of supernatant in an illuminated chamber with a 15 W fluorescent lamp. After this time, 0.12 mM riboflavin final concentration was added and incubated in the same illuminated chamber for an additional 20 min. Absorbance was recorded at 560 nm.

Glutathione reductase

GSH reductase was determined by the method of Carlberg and Mannervik [6]. The rate of oxidation of NADPH by GSSG at 30°C was used as a standard measure for enzymatic activity. The reaction systems of 1 ml contained 1 mM GSSG, 0.1 mM NADPH, 0.5 mM EDTA, 0.1M sodium phosphate buffer (pH 7.6), and a suitable amount of GSH reductase or cytosolic fraction added to give a change in absorbance of 0.05 to 0.30/min. Changes in optical density at 350 and 380 nm were measured. The oxidation of 1 μmol of NADPH/min under this condition was used as a unit of GSH reductase activity. The specific activity was expressed as units/mg protein.

PBS = phosphate buffered saline
MDA = malondialdehyde
NBT = nitro-blue tetrazolium

Catalase

Catalase activity was determined by the Beer and Sizer assay [7] in which the disappearance of H_2O_2 is followed spectrophotometrically at 240 nm. The decrease in absorbance for 2–3 min was recorded in 0.1 ml cytosolic fraction with 1.9 ml deionized water and 1 ml of H_2O_2 (0.059 M) in 0.05 M of potassium phosphate, pH 7. The specific activity was expressed as units/mg protein/min.

Preparation of RNA and PCR amplification

After medium removal, cells were put on ice and washed twice with ice-cold PBS. Total cellular RNA was isolated by lysing the cells with 1 ml trizol as described by Chomczynski and Sacchi [8]. RNA was treated with chloroform, centrifuged (12,000 rpm, 15 min, 4°C) and finally precipitated with ethanol. RNA was redissolved with diethylpyrocarbonate-treated water and its concentration was determined by measuring the optical density at 260 nm. Total RNA (1.5 μg) was used in the reverse transcription reaction with 0.5 μg of oligo dT16 (Perkin Elmer), 10 mM of each of the four deoxynucleotide triphosphates, 25 mM $MgCl_2$, 10 U RNase inhibitor and 50 U of reverse transcriptase (Perkin Elmer) according to the manufacturers instructions. Resulting cDNA was divided into aliquots for PCR amplification of the cDNA products using various sets of sense and antisense primers, and 2 units of Taq polymerase (GibcoBRL). The sequences for the primers were as follows:

IL-1β
(sense: 5'GGATATGGAGCAACAACAAGTGG3',
antisense: 5'ATGTACCAGTTGGGGGAACTG3'),
IL-6
(sense:5'TCAATGAGGAGACTTGCCTG3',
antisense: 5'GATGAGTTGTCATGTCCTGC3'),
IL-8
was performed with upstream primer 5-TTG GCA GCC TTC CTG ATT-3 and downstream primer 5- AAC TTC TCC ACA ACC CTC TG-3 TNF-α
(sense: 5'ACAAGCCTGTAGCCCATGTT3',
antisense: 5'AAAGTAGACCTGCCCAGACT3'),
TGF-β_1
(sense: 5'TTTCGCCTTAGCGCCCACTG3',
antisense: 5'TCCAGCCGAGGTCCTTGCGG3'),
β_2-μglob (sense: 5'CCAGCAGAGAATGGAAAGTC3',
antisense: 5'GATGCTGCTTACATGTCTCG3').

Amplification of TNF-α, IL-1β and IL-6 was at 94°C for 1 min, 72°C for 1 min for 30 cycles. In the case of IL-8 it was 74°C 1 min, 63°C 1 min, and 92°C 1 min for 35 cycles. PCR for β_2-microglobulin was performed with a temperature profile of 74°C for 1 min, 53°C 1 min, and 92°C 1 min for 35 cycles. The PCR products were electrophoresed in 1% agarose gels containing 0.05 μg/ml ethidium bromide. The mRNA IL-8 expression was quantitated using a phospho-imager and accompanying ImageQuant software, and was standardized to the β_2 microglobulin housekeeping gene signal to correct for any variability in gel loading.

Quantitative PCR for β_2-microglobulin, TNF-α, IL-1β and IL-6

To quantitate the mRNA for TNF-α, IL-1β and IL-6, a known amount (0.025 pg) of a standard RNA was added to 1 μg of HepG2 cells RNA before production of cDNA. The standard RNA was produced from plasmid pQA1, which contains a tandem array of primers for β_2-microglobulin and TNF-α, IL-1β or IL-6. The standard RNA serves as an internal control for the reverse transcription reaction and permits generation of standard curves in order to quantitate the specific target mRNAs. The size of the amplicon product derived from the standard RNA differs from the size of the sample-tested RNA. This allows comparison of a known amount of standard RNA with the unknown amount of mRNA in the sample by densitometry. In all samples, a standard curve (including at least four plasmid concentrations) was done for $\beta_2$microglobulin and for TNF-α, IL-1β and IL-6. Samples containing HepG2 cDNA and standard cDNA were amplified as mentioned above. The results were expressed as the number of mRNA molecules of TNF-α, IL-1β and IL-6 per $5x10^4$ molecules of β_2-microglobulin.

Semiquantitation of IL-8 and TGF-β_1 mRNA expression RT-PCR for IL-8 and TGF-β_1 were performed as described above (without plasmid pQA1) using 1 μg of total HepG2 RNA. Amplification products were electrophoresed in parallel with the amount of RNA that contains $5x10^4$ molecules of β_2-microglobulin. Results are expressed as arbitrary units for each sample, as the ratio between the molecule studied and β_2-microglobulin and intensity detected by densitometric analysis.

Immunoassay quantitative determination of TNF-α, IL-1β, IL-6, IL-8 and TGF-β_2

TNF-α, IL-1β, IL-6 and IL-8 were determined by the Quantikine Human TNF-α, IL-1β, IL-6 and IL-8 immunoassay (R&D Systems). A monoclonal antibody specific for TNF-α, IL-1β, IL-6 and IL-8 was pre-coated onto a microtiter plate. Standards and samples were pipetted into the wells, where TNF-α IL-1β, IL-6 or IL-8 were bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TNF-α, IL-1β, IL-6 or IL-8 was added to the wells. After a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The color development was stopped and the intensity of the color measured at 450 nm spectrophotometrically.

TGF-β_1 was determined by a TGF-β_1 Quantikine kit (R&D systems). To activate the latent form to immunoreactive TGF-β_1, 0.5 ml of culture medium was incubated for 10 min at room

PCR = polymerase chain reaction

RT-PCR = reverse transcriptase polymerase chain reaction

Table 1. Neutral red, GSH content, lipid peroxidation damage, and SOD, GSHPx and catalase activity of HepG2 cells treated for 24 hours with 50 mM ethanol, 175 μM acetaldehyde or 1 μg/ml LPS

Treatment	Neutral red (% control)	GSH (nmol mg protein)	Lipid peroxidation (pmolMDA/mg protein)	SOD (U/mg protein)	GSHPx (U/mg protein)	Catalase (U/mg protein)
Control	100	202±24	2,866±798	1.1±.06	26±9	664±39
Ethanol	83±4	138±32	5,233±1,012	0.8±.04	9±2	440±13*
Acetaldehyde	59±4*	116±14	10,300±1,117*	0.7±.02	9±4	350±22*
LPS	92±7	144±6	5,633±834	0.8±.01	15±7	441±45*

Values represent mean SE for three independent experiments carried out in triplicate. *Significantly different from control ($P < 0.05$).

temperature with 0.1 ml 1N HCl and then neutralized by adding 0.1 ml 1.2 N NaOH/0.5M HEPES. Standard or culture medium (200 μl) was incubated in the microtiter plate covered by TGF-β_1 soluble type II receptor that binds TGF-β_1. After washing each well three times with a buffer solution, 200 μl of polyclonal antibody against TGF-β_1 conjugated to horseradish peroxidase was added and incubated for 1.5 hour at room temperature. The media were aspirated and washed. After a 20 min incubation with 200 μl of substrate solution (hydrogen peroxide and tetramethylbenzidine) the reaction was stopped with 50 μl H_2SO_4 2N. The optical density of each well was determined within 30 min using a microtiter plate reader set to 450 nm.

Data analysis

Data are reported as mean ES. The SPSS package version 8 was used to run the analysis. ANOVA was used for comparison among groups, and Tukeys method for multiple comparisons. $P < 0.05$ was considered as statistically significant.

Results

HepG2 cell were treated with ethanol, acetaldehyde and LPS for 24 hours; and neutral red assay, lipid peroxidation, glutathione content, antioxidant enzymes and expression and secretion of IL-1β, IL-6, IL-8, TNF-α and TGF-β_1 were determined. Under all experimental conditions tested, cells presented 100% viability by trypan blue assay (data not shown). Lysosomal capacity, determined by neutral red assay, only diminished with acetaldehyde treatment [Table 1]. There was no indication of any change in total SOD or glutathione peroxidase 24 hours after ethanol, acetaldehyde or LPS treatment. Catalase activity diminished with all treatments, 34% with ethanol and LPS, while acetaldehyde produced a 47% decrease [Table 1]. Ethanol, acetaldehyde or LPS treatment failed to alter the intracellular levels of reduced GSH [Table 1]. Lipid peroxidation value increased by 260% in acetaldehyde-treated cells as compared to control cells, while ethanol and LPS did not produce a significant increase in this parameter [Table 1].

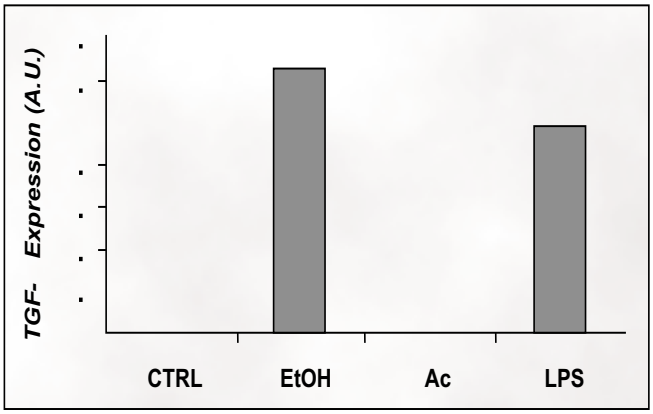

Figure 1. Quantitative TNF-α gene expression in HepG2 cells treated for 24 hours with 50 mM ethanol, 175 μM acetaldehyde and 1 μg/ml LPS. RNA was isolated and subjected to RT-PCR as described in Materials and Methods. The results were expressed as the number of mRNA molecules of TNF-α per $5x10^4$ molecules. Two quantifications were done for each experimental condition.

Non-treated HepG2 cells (controls) did not express or secrete TNF-α, IL-1β, IL-6 or TGF-β_1, and only IL-8 mRNA was expressed and secreted. Induction of TNF-α expression was observed with ethanol (3,500 mol/$5x10^4$ mol β_2-μglob), acetaldehyde (18,700 mol/$5x10^4$ mol β_2-μglob) and LPS (2,502 1mol/$5x0^4$ mol β_2-μglob) [Figure 1]. Expression of IL-6 was induced solely by LPS (1,346 mol/$5x10^4$ mol β_2-μglob), and TGF-β_1 expression was detected only after exposure to ethanol (1.25 AU) and LPS (0.98 AU) [Figure 2]. IL-1β mRNA was not detected. IL-8-mRNA expression did not differ from control cells in ethanol, acetaldehyde or LPS treated HepG2.

A slight secretion of IL-1β was induced by ethanol, acetaldehyde and LPS. TGF-β_1 was secreted after exposure to ethanol and LPS. IL-6 secretion was induced only by LPS (14.3±2 pg/ml$x10^6$ cells). No secretion of TNF-α was detected. HepG2 cells readily responded to acetaldehyde and LPS, with secreted IL-8 increasing 57% and 50% respectively as compared to the control value [Table 2].

Discussion

These studies provide evidence that hepatocytes are capable of producing cytokines and oxidative stress damage in response to specific stimuli, and that this response is both qualitatively and quantitatively dependent on the specific stimulant. The findings of the present study indicate that HepG2 cells under aggression

AU = arbitrary units

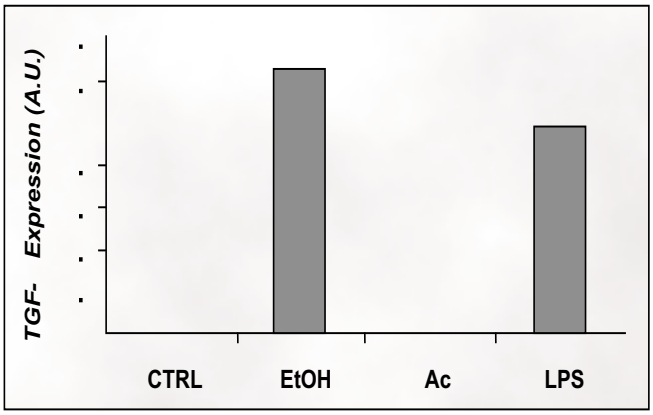

Figure 2. Semi-quantitative mRNA for TGF-β_1 from HepG2 cells treated for 24 hours with 50 mM ethanol, 175 μM acetaldehyde and 1 μg/ml LPS. RNA was isolaled and subjected to RT-PCR as described in Materials and Methods. The TGF-β_1-mRNA was quantitated using phospho-imager equipment, standardized to and expressed as arbitrary units (AU).Two quantifications were done for each experimental condition.

by acetaldehyde presented lipid peroxidation damage. Ethanol did not increase this parameter due to the fact that these cells could not metabolize ethanol under the experimental conditions tested. Treatment with ethanol, acetaldehyde and LPS decreased catalase activity, and probably as a consequence led to an increase of hydrogen peroxide inside the cell. No change in the other antioxidant enzyme activities was observed. HepG2 cells display a differential cytokine response: induction of mRNA TNF-α with ethanol, acetaldehyde and LPS, although no secretion was observed. Neuman et al. [9] observed up-regulated expression and secretion in HepG2 cells treated with 80 mM ethanol. No TNF-α secretion was reported with lower ethanol concentration. It would be of interest in the future to design experiments to explore a dose-effect relationship and establish the threshold concentration of ethanol to secrete TNF-α. TGF-β_1 expression and secretion were detected only after ethanol and LPS exposure. IL-1β expression was not detected after 24 hour treatment with ethanol, acetaldehyde and LPS, but a slight secretion was measured in the culture media. IL-1β is one of the early damage mediators and its mRNA degradation might take place before the determination. Expression and secretion of IL-6 were observed only after LPS treatment. IL-6 enhanced the formation and release of LPS-binding protein by hepatocytes, which is an important event in the signaling sequences that sensitize liver tissue to endotoxemia associated with ethanol ingestion [10]. Our findings demonstrate that HepG2 cells, in a basal way, express mRNA and secrete IL-8. This is a critical factor in the recruitment of neutrophils to sites of inflammation and oxidant stress and an important regulator of its expression. IL-8 mRNA expression in cells treated with ethanol, acetaldehyde and LPS did not differ as compared to control

Table 2. TGF-β_1, IL-1β and IL-8 secretion from HepG2 cells treated for 24 hours with 50 mM ethanol, 175 μM acetaldehyde or 1 μg/ml LPS

Cytokine ($pg/10^6$ cells) Treatment	TGF-β_1	IL-1β_1	IL-8
Control	0	0	141 ± 10
Ethanol	199 ± 6*	8.6 ± 0.9*	196 ± 11.5
Acetaldehyde	0	11 ± 0.9*	223 ± 21*
LPS	380 ± 22*	7.2 ± 0.8*	213 ± 18.9*

Values represent mean SE for three independent experiments carried out in triplicate. *Significantly different from control ($P < 0.05$).

cells. However, HepG2 cells readily responded to acetaldehyde and LPS and secreted a greater quantity of IL-8 than control or ethanol-treated cells. Our data show that acetaldehyde, but not ethanol, is responsible for IL-8 secretion in cases of alcohol abuse. The negative results are not surprising in view of other reports suggesting that ethanol has either no effective or a suppressive effect on IL-8 production by human and rat parenchymal cells.

Due to the change in the redox potential of a cell following ethanol, acetaldehyde and LPS treatment, it is likely that the toxin directly affects transcription and translation of genes via oxidative or reductive interactions including DNA damage and protein degradation. Moreover, transcription factors such as nuclear factor-κB and activating protein are sensitive to ROS production and pro-oxidant conditions and are thought to play a key role in the development of alcoholic liver disease through up-regulation of inflammatory cytokines. Decreasing catalase activity indicates an increased propensity for cellular protein regulation via ROS.

The present research confirms and extends the notion that parenchymal cells not only respond to cytokines but also secrete them in response to hepatic injury of various stimuli. Furthermore, cytokine production by hepatocytes involves selective signaling processes. The exact role of hepatocyte-derived cytokines in liver injury is unknown. The change in the redox potential may indirectly influence transcription and translation through a secondary mechanism such as cytokine production and alter the activity of transcription factors.

Our data illustrate the potential usefulness of an *in vitro* model to delineate mechanisms that participate in the development of alcoholic liver disease.

Acknowledgements: This work was supported by Consejo Nacional de Ciencia y Tecnologia (CONACYT) No. 400200-5-25175M.

References

1. Lands WEM. Cellular signals in alcohol induced liver injury. A review. *Alcohol Clin Exp Res* 1995;19:928–38.
2. Diehl AM. Effect of ethanol on tumor necrosis factor signaling during liver regeneration. *Clin Biochem* 1999;32:571–8.
3. Borenfreund E, Puerner, JA. Cytotoxicity of metals, metal-metal, and metal-quelator combinations in vitro. *Toxicology* 1985;39:121–34.

4. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978;52:302–10.
5. Tietze F. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:507–22.
6. Carlberg I, Mannervil B. Purification and characterization of the flavo-enzyme glutathione reductase from rat liver. *J Biol Chem* 1975;250:5475–80.
7. Beer RF, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J Biol Chem* 1952;195:133–40.
8. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156–9.
9. Neuman MG, Shear NH, Bellentani S, Tiribelli C. Role of cytokines in ethanol-induced cytotoxicity in vitro in HepG2 cells. *Gastroenterology* 1998;115:157–66.
10. Watson AM, Warren G, Howard G, Shedlofsky SI, Blouin RA. Activities of conjugating and antioxidant enzymes following endotoxin exposure. *J Biochem Molec Toxicol* 1999;13:63–9.

Correspondence: Dra. Ma. Concepcion Gutierrez-Ruiz, Departamento de Ciencias de la Salud, Division de Ciencias Biologicas y de la Salud, Universidad Autonoma Metropolitana-Iztapalapa, Apdo Postal 55-535, Mexico, D.F. 09340, Mexico. Phone: (52-5) 804-6451, Fax: (52-5) 804-4730, email: mcgr@xanum.uam.mx