BIOMEDICAL AND ENVIRONMENTAL SCIENCES **17**, 315-326 (2004)

Induction of Heme Oxygenase-1 in Human Hepatocytes to Protect Them From Ethanol-induced Cytotoxicity[1]

LIE-GANG LIU[*,2], HONG YAN[*], WEN ZHANG[*], PING YAO[*], XI-PING ZHANG[*], XIU-FA SUN[*], AND ANDREAS K. NUSSLER[§]

[*]Department of Nutrition and Food Hygiene, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China; [§]Department of General, Visceral, and Transplantation Surgery, Humboldt University, Charité, Campus Virchow, Augustenburger Platz 1, 13353 Berlin, Germany

Background/Aim We investigated the relationship between ethanol exposure and heme oxygenase (HO-1) in human hepatocytes in order to ascertain if induction of HO-1 can prevent ethanol induced cellular damage. **Methods** Dose-dependent (25-100 mmol/L) and time-dependent (0-24 h) ethanol exposure were used in the present study. HO-1 mRNA and protein expression were detected by PT-PCR and Western blot respectively. HO-1 activity was indicated by bilirubin and Fe^{2+} formation. Cytotoxicity was investigated by means of lactate dehydrogenate (LDH) and aspartate transaminase (AST) level in culture supernatants, as well as the intracellular formation of malondialdehyde (MDA), cellular glutathione (GSH) status and CYP 2E1 activity. **Results** We first demonstrated a dose-dependent response between ethanol exposure and HO-1 mRNA and protein expression in human hepatocytes. We further observed a time-dependent increase of HO-1 mRNA expression using 100 mmol/L ethanol starting 30 minutes after ethanol exposure, reaching its maximum between 3 h and 9 h. Being similar to what had been demonstrated with the mRNA level, increased protein expression started at 6 h after ethanol exposure, and kept continuous elevated over 18 h. In addition, we found that ethanol exposure to hepatocytes markedly increased HO-1 enzyme activity in a time-dependent manner measured as bilirubin and Fe^{2+} formation in human hepatocytes. Our results clearly showed that ethanol exposure caused a significant increase of LDH, AST, and MDA levels, while the antioxidant GSH was time-dependently reduced. Furthermore, we demonstrated that pre-administration of cobalt protoporphyrin (CoPP) induced HO-1 in human hepatocytes, and prevented an increase of MDA and a decrease of GSH. These effects could be partially reversed by zinc protoporphyrin (ZnPP), an antagonist of HO-1 induction. **Conclusion** HO-1 expression in cells or organs could lead to new strategies for better prevention and treatment of ethanol-induced oxidative damage in human liver.

Key words: Heme oxygenase-1; Ethanol; Cellular stress; Human hepatocytes

---

[1]This work was supported by the National Natural Science Foundation of China (NSFC) (30271130).

[2]Correspondence should be addressed to Lie-Gang LIU, Ph. D., Department of Nutrition and Food Hygiene, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Wuhan, 430030, Hubei, China. Tel: 86 (0)2783692711. Fax: 86 (0)2783693307. E-mail: lgliu@mails.tjmu.edu.cn

Biographical note of the first author: Lie-Gang LIU, male, born in 1966 China, Ph. D., associate professor, majoring in molecular toxicology.

Abbreviations: AST, aspartate transaminase; CoPP, cobalt protoporphyrin; CYP-450, cytochrome P-450; dNTP, dideoxynucleotide triphosphates; DTT, dithiothreitol; GSH, glutathione; HEPES, N-[2-hydroxythy] piperazine-N´2-[2-ethanesulfonic acid]; HO, heme oxygenase; LDH, lactate dehydrogenate; MDA, malondialdehyde; NADP, nicotide adenine dinucleotide phosphate; NF-κB, nuclear factor - kappa B cell; PCR, polymerase chain reaction; ROS, reactive oxygen species; ZnPP, zinc protoporphyrin.

0895-3988/2004
CN 11-2816

INTRODUCTION

Alcohol abuse and its associated illnesses are among the most common problems worldwide, causing serious medical, financial and social losses. Alcohol-induced liver injury progresses from fatty infiltration and follows a harmful course of inflammation leading to irreversible damage. The most severe disorder is cirrhosis, and liver transplantation at a cost of approximately $100 000 each, seems to be the only cure[1]. Although the histophathological changes of early alcohol liver disease, namely steatosis, inflammation and necrosis, have been well documented, the exact pathogenesis of this devastating disease is still unknown. The elucidation of alcohol mechanisms has resulted in a better understanding of the pathophysiology of alcohol-induced liver damage and may eventually provide new strategies for better prevention and treatment.

Ethanol is mainly metabolized in the liver by alcohol dehydrogenases to form acetaldehyde and further metabolized to form acetate. Ethanol can also be metabolized by catalase and more selectively by cytochrome P-450 2E1 (CYP2E1). Acetaldehyde is the first oxidation product of ethanol. Due to high reactivity it is responsible for many aspects of alcohol related liver injury. It is generally recognized that lipid peroxidation, immune damage and antioxidant defenses may play an important role in the pathogenesis of ethanol-induced cellular injury[2].

The rate-limiting enzyme in heme catabolism, heme oxygenase (HO), is a protein that catalyzes the degradation of toxic heme to biliverdin and Fe^{2+} with the concurrent release of carbon monoxide (CO)[3]. Biliverdin is then further converted to bilirubin by the cytosolic enzyme, biliverdin reductase. HO exists in form of three isoenzymes[4]. HO-2, which is expressed constitutively, is present in high concentrations in tissues such as brain and testis. Recently, McCoubrey *et al*. described a 33-kDa protein encoding for HO-3, however its exact function is not clear yet[5]. HO-1, which seems to be ubiquitously distributed in mammalian tissues, is strongly and rapidly induced by a variety of stimuli, such as reactive oxygen species, heavy metals, irradiation, chemotherapeutic agents, GSH depletion, endotoxin, hyperoxia, hormones, various cytokines, or even by heme itself[6].

Although the function of this enzyme is still not completely understood, increasing evidence strongly suggests that induction of HO-1 provides potent cytoprotective effects on various *in vitro* and *in vivo* model of oxidative damages and stresses[6]. This evolving hypothesis is further supported by observations made in HO-1-deficient mice and humans[7,8]. The mechanism by which HO-1 can mediate these cytoprotective functions is not clear. However, three major catalytic by-products, CO, ferritin and bilirubin may represent potential targets to prevent cellular damage[9,10].

Ethanol exposure is considered as an oxidative stress. So far there is only little evidence that ethanol exposure induces HO-1 activity in rats and mice[11,12]. According to our knowledge there is no proof whether or not ethanol exposure would affect HO-1 enzyme activity in humans, especially in ethanol metabolizing liver cells. Therefore, the following questions were raised in the present work. Did ethanol exposure increase HO-1 mRNA and protein expression as well as HO-1 enzyme activity in human hepatocytes? Did HO-1 induction protect human hepatocytes from ethanol exposure?

In our experiments a dose-dependent (25-100 mmol/L) and time-dependent (0-24 h) ethanol exposure were used to investigate the relationship between ethanol exposure and HO-1 expression. Cytotoxicity was investigated by means of LDH, AST activity, as well as MDA formation and changes in cellular GSH status.

MATERIALS AND METHODS

Materials

Williams' medium E (with glutamax-1), HEPES, and penicillin/streptomycin were obtained from Life Technologies (Karlsruhe, Germany). Insulin and hydrocortisone were supplied from Sigma (Deisenhofen, Germany), while calf serum was purchased from PAA Ltd. (Linz, Austria). Western blot development kits were purchased from Amersham (ECL, Buckinghamshire, UK). Heme oxygenase-1 mouse-monoclonal IgG1 antibody was purchased from Transduction Laboratories, Lexington, KY. All other reagents were obtained from Sigma unless otherwise indicated.

Isolation of Human Hepatocytes and Cell Culture

Human liver tissue was obtained from liver resections of tumor patients with primary or secondary liver tumors. The collection of tissue was done according to institutional guideline and with patients' written consent. Immediately after resection, a wedge section of the normal tumor-free tissue was transferred under sterile conditions to the laboratory in culture media. Human hepatocytes were isolated by a two-step collagenase perfusion technique followed by a Percoll centrifugation step as previously described[12]. Hepatocyte purity assessed under light microscopy, was over 95% and viability consistently exceeded 93% by trypan blue exclusion. The freshly harvested human hepatocytes were then seeded onto rat-tail collagen-coated Petri dishes or 6-well culture trays. The medium consisted of Williams' E medium supplemented with 1 μmol/L insulin, 15 mmol/L HEPES, 1.4 μmol/L hydrocortisone, 10% calf serum, and penicillin/streptomycin (100 U/100 μg/mL). Cells were incubated in a humidified incubator in an atmosphere containing 95% air and 5% CO_2 at 37℃ until cell attachment. On the following day, cells were exposed to various concentrations of ethanol (25 to 100 mmol/L) for 9 h. Time-dependent expression studies were undertaken in human hepatocytes using 100 mmol/L ethanol between 0.5 to 24 h. At the corresponding time intervals cells and supernatants were collected in agreement with the applied technique. In additional experiments, cells were incubated with 5 μmol/L CoPP to induce HO-1 or with 0.5 μmol/L ZnPP to antagonize the effects of HO-1.

Reverse Transcriptase-polymerase Chain Reaction

Total RNA was isolated according to the method of Chomczynski and Sacchi[13]. Ten μg of total RNA was reversely transcribed into cDNA in 30 μL reaction mixture containing Superscript IIRT, dNTP and Oligo $(dt)_{12\text{-}18}$ primers. cDNA was used to detect HO-1 mRNA by PCR using specific primers. Primers for HO-1 were designed on the basis of the human HO-1 cDNA sequence: 5′-TGC GGT GCA GCT CTT CTG 3′ (anti-sense) and 5′-GCA ACC CGA CAG CAT GC-3′ (sense), amplifying a product of 244 bp at cycles 35[14]. PCR reactions were carried out in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT) in a total volume of 20 μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3) 50 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 U Taq polymerase, and 200 μmol/L dNTPs each. The amplification procedure was as follow: initial denaturation of the template at 96℃ for 3 min; then 35 amplification cycles were carried out at 96℃ for 30 sec (denature), at 50℃ for 30 sec (annealing), and at 72℃ for 90 sec (extension) followed by a final extension at 72℃ for 10 min. Equal loading was verified by β-actin expression, using specific primers as described before: 5′-ACC CAC ACT GTG CCC ATC TA-3′ (anti-sense) and 5′-CGG AAC CGC

TCA TTG CC-3′ (sense) amplifying a 289-bp PCR production[15]. PCR products were electrophoresed on 1.5% agarose gels containing 0.1 μg ethidium bromide and photographed under UV transillumination.

Western Blot Analysis

Cells were washed two times with PBS and homogenized in buffer containing protease inhibitors. Protein concentrations were determined by the method of Lowry, using bovine serum albumin as the standard. Proteins were separated on a 12.5% SDS polyacrylamid gel, and then transferred onto polyvinylidene difluoride membranes[12,16]. Nonspecific binding sites were blocked by overnight incubation of membranes in 5% nonfat milk solution solved in PBS/Tween-20 at 4℃. After washed with PBS/Tween-20, the membranes were incubated with heme oxygenase-1 mouse-monoclonal IgG1 antibody, followed by an incubation with horseradish-peroxidase-conjugated anti-mouse antibody at room temperature for 1 h. Then, the membranes were washed again with PBS/Tween-20 for 1 h, and the immune complexes were developed using a chemiluminescence detection system. Equal loading of total protein was verified using commercially available antibody against β-actin[17].

HO -1 Enzyme Activity

HO-1 enzyme activity was assessed by two methods, namely conversion of heme to bilirubin[18] and formation of intracellular Fe^{2+}[16]. Formation of bilirubin was measured as follows. Briefly, 20 000×g supernatants was prepared from untreated and treated hepatocytes incubated either with 5 μmol/L CoPP or various concentrations of ethanol. Then, study samples were mixed with 2 mg/mL human liver cytosol (100 000×g supernatant, as a source of biliverdin reductase), 1 mmol/L $MgCl_2$, 3 units of glucose-6-phosphate dehydrogenase, 1 mmol/L glucose-6-phosphate, and 2 mmol/L $NADP^+$ solved in 0.5 mL of 0.1 mol/L potassium phosphate buffer, pH 7.4 for 30 min at 37℃ in the dark in a shaking water bath. Amounts of generated bilirubin were estimated using a scanning spectrophotometer, and defined as the difference between 450 and 530 nm with an extinction coefficient for bilirubin of 40 mmol/L • cm^{-1}. The wavelength of bilirubin was at a maximum of 467 nm. Results were expressed as pmoles bilirubin/mg protein/min. Intracellular Fe^{2+} levels were determinated using a standard analytic procedure as recently described[17]. Absorbency was measured spectrophotometrically at 550 nm and results were expressed as μmoles/mg protein.

Cellular Damage

Lactate dehydrogenase (LDH) and aspartate transaminase (AST) measurement The increase in LDH activity was seen under many pathologic conditions. Release of the intracytoplasmic enzyme LDH into cell culture medium was frequently used as a measure of cellular injury. AST was only present in hepatocytes, located in mitochondria and cytoplasm. Like LDH, its increase was an early sign of cellular injury. Both enzymes were measured using a commercially available test kit from Sigma (Deisenhofen, Germany). Results were expressed as units per liter (U/L).

Glutathione (GSH), malondialdehyde (MDA), and CYP2E1 measurement Total cellular GSH content was assessed according to the method of Akerboom and Sies[19]. Absorbance was measured spectrophotometrically at 405 nm. Results were expressed as nmoles/mg of protein. MDA, a by-product of lipid peroxidation, was assayed in human hepatocytes using the thiobarbituric acid reaction as described by Wright *et al.*[20]. Absorbance of the resulting organic layer was measured spectrophotometrically at 532 nm and calculated according to

an external standard (1,1,3,3-tetraethoxypropane) of MDA. Results were expressed as nmoles/mg of protein. Cytochrome P450 2E1 was induced by a broad variety of chemicals, such as ethanol. The increase of reactive oxygen species by CYP2E1 induction seemed to play an important role in ethanol-induced hepatocellular damage[21]. Activity of CYP2E1 was determined by the rate of hydroxylation of p-nitrophenol, at 546 nm[22]. The extinction coefficient for p-nitrophenol was 10.28 mmol/L • cm. Results were expressed as formed p-nitrophenol nmoles/min per mg protein.

Statistical Analysis

Values were expressed as $\bar{x}\pm s$. Significances were determined by using the ANOVA test. Statistical significance was established at P value < 0.05.

RESULTS

Dose and Time-dependent HO-1 mRNA Expression by Ethanol

A dose-dependent response between ethanol exposure and HO-1 mRNA expression was observed in human hepatocytes. Fig. 1A shows a sustained dose-dependent increase of HO-1 mRNA expression when cells were exposed to various concentrations of ethanol. Based on these results, we chose 100 mmol/L ethanol to investigate the time-dependent HO-1 mRNA expression. As shown in Fig. 1B, 100 mmol/L ethanol led to a continuous increase in HO-1 mRNA expression starting as early as 30 minutes after exposure, reaching its maximum between 3 h and 9 h. Thereafter, HO-1 mRNA levels continuously decreased.

Dose and Time-dependent HO-1 Protein Expression After Ethanol Exposure

Fig. 2A shows a dose-dependent expression of HO-1 protein in human hepatocytes after 9 h of ethanol exposure. Being similar to what was demonstrated on the mRNA level, the lowest increase in HO-1 protein expression was seen in human hepatocytes at the lowest ethanol (25 mmol/L) dose, and continuously increased by enlarging the ethanol dose. Fig. 2B demonstrates that the exposure of 100 mmol/L ethanol to human hepatocytes continuously increased the HO-1 protein expression starting at 6 h during the investigation period.

Dose and Time-dependent HO-1 Enzyme Activity by Ethanol

The above experiments clearly showed that ethanol itself could induce HO-1 mRNA and protein expression. In the following set of experiments we addressed the questions if the incubation of hepatocytes with ethanol would also stimulate HO-1 enzyme activity. As shown in Fig. 3A, we observed a dose-dependent increase in HO-1 enzyme activity reaching its maximum at 100 mmol/L ethanol after 9 h exposure as compared to untreated control cultures (CT: 7.2±0.32 pmol/mg protein/minute vs. 100 mmol/L ethanol: 12.86±0.63 pmol/mg protein/minute; P<0.05 vs. CT). As shown in Fig. 3B, we found that the exposure of 100 mmol/L ethanol led to a continuous increase in HO-1 enzyme activity in human hepatocytes. HO-1 enzyme activity increased rapidly and reached its maximum between 9-12 h after ethanol exposure. Then, by 24 h we saw a decline of HO-1 enzyme activity. HO-1 enzyme activity between 6 and 24 h was significantly higher compared to untreated control cultures (*P<0.05 vs. CT).

HO-1 was known to degrade toxic heme into various products such as biliverdin, carbon monoxide and Fe^{2+}. Therefore, we attempted to measure intracellular changes of Fe^{2+}

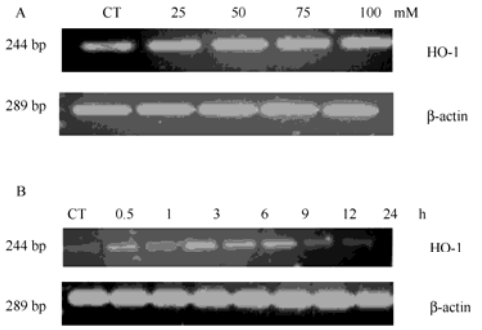

FIG. 1A. Dose-dependent induction of HO-1 mRNA expression in human hepatocytes by ethanol. Human hepatocytes were incubated with medium alone or medium containing various concentrations of ethanol (25-100 mmol/L) for 9 h. Total RNA was analyzed for HO-1 mRNA expression by RT-PCR as described. B. Time-dependent induction of HO-1 mRNA expression. Hepatocytes were incubated with 100 mmol/L ethanol for various length of time (0-24 h). Total RNA was analyzed for HO-1 mRNA expression by RT-PCR as described.

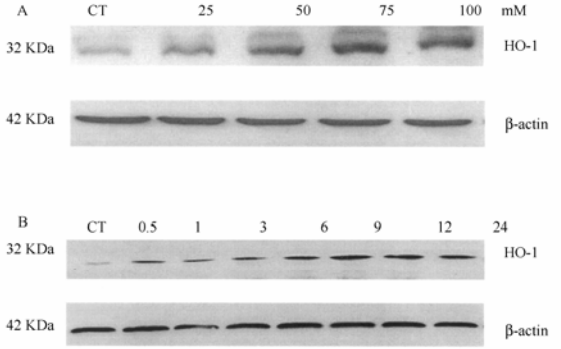

FIG. 2A. Ethanol dose-dependent induction of HO-1 protein expression Western blot analyzes. Hepatocytes were incubated with various concentrations of ethanol (0-100 mmol/L) for 9 h. 20 000 g cytosol was prepared and 100 μg sample was loaded each. B. Time-dependent induction of HO-1 protein expression in human hepatocytes after 100 mmol/L of ethanol exposure (0-24 h). 20 000 g cytosol was prepared and 100 μg sample was loaded each. β-actin is shown for equal loading.

and questioned if the changes could be indirectly linked to ethanol-induced HO-1 enzyme activity in human hepatocytes. As shown in Fig. 4, we found a time-dependent intracellular Fe^{2+} release starting 1h after ethanol exposure which was however insignificant ($P > 0.05$ vs. CT). The iron release continued to increase over the investigation period and reached its maximum at 24 h. A significant difference between ethanol-treated and untreated cell cultures was observed between 9 h and 24 h ($P < 0.05$ vs. CT). Our results underlined that both formation of bilirubin and release of Fe^{2+} could be used to measure HO-1 enzyme activity in human hepatocytes.

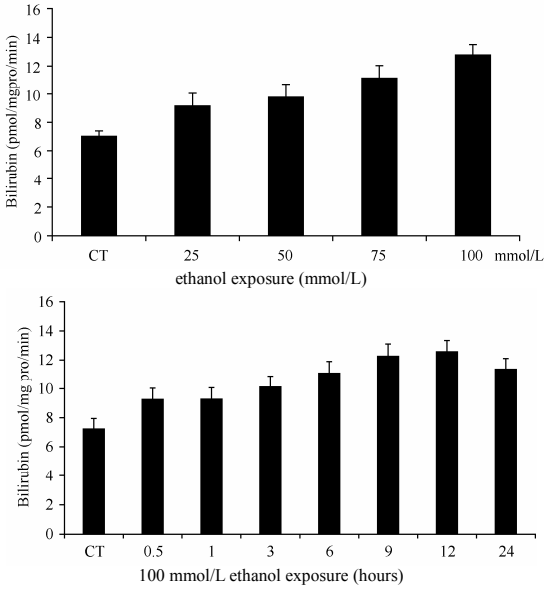

FIG. 3A. Ethanol-dependent HO-1 enzyme activity measured as bilirubin formation. Hepatocytes were incubated with cell culture medium in the presence or absence of various ethanol concentrations for 9 h. 20 000 g cytosol was prepared and bilirubin formation was measured as described. $^{*}P<0.05$ vs. control cultures. B. Time course of 100 mmol/L ethanol exposure on bilirubin formation. Hepatocytes were incubated with 100 mmol/L of ethanol for various length of time (0-24 h). 20 000 g cytosol was prepared and bilirubin levels were measured as described. Each point represents $\overline{x} \pm s$ of triplicates of six independent experiments. $^{*}P<0.05$ vs. CT.

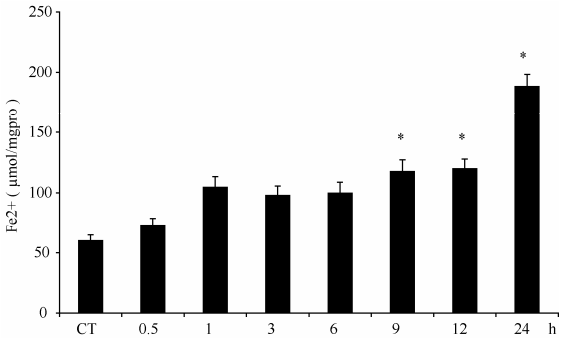

FIG. 4. Time course of iron release after ethanol exposure. Hepatocytes were incubated with 100 mmol/L ethanol over 24 h and the intracellular Fe^{2+} concentrations were measured as described. Each point represents $\overline{x} \pm s$ of triplicates of five different experiments. $^{*}P<0.05$ vs. CT.

Hepatocyte Damage Due to Ethanol Exposure

From Table 1 we observed that human hepatocytes incubated with 100 mmol/L ethanol showed a time-dependent CYP 2E1 increase. The highest degree of enzyme activity was seen at 9 h of ethanol exposure as compared to untreated control cultures (P<0.01 vs. CT). Table 1 also shows that MDA levels increased with time, reaching its significance after 3 h (P<0.05 vs. controls) ethanol exposure. On the other hand, we found a time-dependent decline of cellular GSH levels 6 h after ethanol exposure. Moreover, the degree of cellular injury caused by ethanol could be estimated by the leakage of enzymes from hepatocytes. In order to evaluate the hepatocellular damage caused by ethanol, culture supernatants taken from hepatocyte cultures were screened for the presence of LDH and AST. In our experiments ethanol caused a clear dose-dependent (LDH: CT=16.7±1.96 U/L, 100 mmol/L ethanol: 94.8±10.05 U/L, P<0.05; AST: CT=102.11±11.02 U/L, 100 mmol/L ethanol: 176.71±16.36 U/L, P<0.05 vs. CT) and time-dependent release of LDH and AST (LDH: CT=15.6±1.66 U/L, 100 mmol/L ethanol (24 h):=84.5±7.86 U/L, P<0.05; AST: CT=94.22±9.52 U/L, 100 mmol/L ethanol (24 h)=168.92±16.86 U/L, P< 0.05 vs. CT).

TABLE 1

Hepatocyte Damage Due to Ethanol Exposure

Time（h）	CYP2E1 (pmol/mgpro/min)	MDA (nmol/mg protein)	GSH (nmol/mg protein)
CT	43.20±4.20	36.37±3.64	7.52±0.29
0.5	59.45±5.12	40.54±3.87	7.62±0.47
1	73.84±6.82	44.88±3.52	8.02±0.65
3	77.36±6.53*	58.88±4.06*	6.87±0.69
6	78.22±6.56*	61.64±4.23*	4.12±0.86*
9	103.46±12.52**	69.05±4.41**	2.95±0.45**
12	83.94±7.36**	71.10±3.68**	3.07±0.35**
24	74.56±7.16*	72.11±3.26**	2.50±0.51**

Note. *P<0.05, **P<0.01vs. CT untreated control cultures.

Did HO-1 Induction in Human Hepatocytes Protect Ethanol-induced Oxidative Damage?

In the following set of experiments we addressed the question whether the induction of HO-1 could protect human hepatocytes from ethanol-induced cellular damage. In preliminary results we found that the co-induction of ethanol with 5 μmol/L CoPP, a potent inducer of HO-1, did not lead to cellular protection of ethanol-induced cytotoxicity (data not shown). In contrast, when cells were pre-incubated with CoPP for 8 h and then incubated with ethanol, we saw a profound reduction of MDA formation caused by ethanol (Fig. 5A). This positive effect of CoPP pre-incubation was also seen with regard to cellular GSH levels (Fig. 5B), and a large decrease was seen with regard to the release of LDH and AST (data not shown). Most interesting was that ZnPP, which antagonized the effects of CoPP, could partially reverse its protecting effects on MDA formation and cellular GSH level.

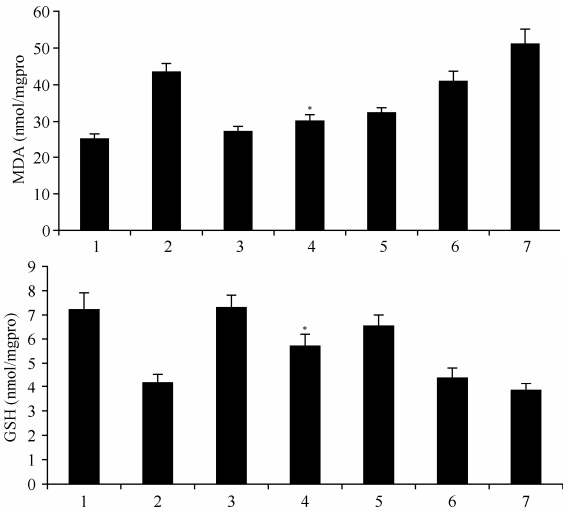

FIG. 5. The induction of HO-1 in human hepatocytes protects against MDA (**A**) formation and cellular GSH (B) reduction. 1: Control, 2: 100 mmol/L ethanol ; 3: 5 μmol/L CoPP, 4: 100 mmol/L ethanol plus 5 μmol/L CoPP, 5: 0.5 μmol/L ZnPP, 6: 100 mmol/L ethanol plus 5 μmol/L CoPP + 0.5 μmol/L ZnPP, 7: 0.5 μmol/L ZnPP + 100 mmol/L ethanol. CT: control; $^{*}P<0.05$ vs. 100 mmol/L ethanol. Hepatocytes were pre-incubated with CoPP, and ZnPP for two hours, then, 100 mmol/L ethanol was added, and cultures were incubated overnight. Next morning, cells were harvested for the measurement of cellular MDA and GSH levels as described. Each point represents the $\bar{x} \pm s$ of triplicates of five different experiments.

DISCUSSION

Ethanol consumption and its effects on human beings have been well studied. However, reports on the influence of the stress gene HO-1 and its possible positive effects on ethanol-induced toxicity are few. The first evidence that ethanol exposure induced HO-1 activity in rats and mice was reported[11]. However, no substantial data on humans are available, especially the interaction of ethanol on liver cells and HO-1 gene expression. To our knowledge, this is the first time that human hepatocytes were used to assess the relationship between HO-1 gene expression and the possible protecting effects of HO-1 induction on cellular damage caused by ethanol. In the present study, 25-100 mmol/L ethanol was used to investigate the relationship between ethanol exposure and HO-1 gene expression. These dosages could lead to sustained cellular damages such as teratogenesis in guinea pigs, ethanol-induced unconsciousness or even death in humans[23].

Biological systems are equipped with an array of anti-oxidative defense systems that can more or less scavenge and/or convert reactive oxygen intermediates (ROI) into harmless species. The anti-oxidative defense system includes enzymes, such as superoxide dismutase, catalase, glutathione peroxide, glutathione reductase, tripeptide glutathione, or vitamins A, C, and E, all of which can scavenge and/or limit toxic effects of ROI. An imbalance in ROI/free radicals and antioxidants would cause oxidative stress within cellular systems. The toxic effects of ethanol arise mainly from the formation of acetaldehyde, but ethanol toxicity

is also dependent on free radical-mediated toxicity, which is related to the decrease of cellular glutathione levels or increase of lipid peroxidation in hepatocytes. In the present study CYP 2E1 activity, cellular GSH and MDA levels were chosen to assess hepatocyte damage caused by ethanol exposure.

CYP 2E1 could be induced by a broad variety of chemicals, such as ethanol. The production of reactive oxygen species via CYP2E1 induction could contribute to the development of alcoholic liver disease or at least increase of its cytotoxic effects[24]. Ethanol is an essential CYP 2E1 inducer in human hepatocytes, which was also confirmed in a recent study by Ponsoda *et al*.[25], showing that human hepatocytes incubated with 100 mmol/L ethanol showed a 2- to 3- fold increase in CYP 2E1 enzyme activity compared with untreated control cultures. Using recombinant retroviral expression, in addition to ethanol (20-100 mmol/L) exposure, the culture medium of HepG2 cell line resulted in both a large increase of CYP 2E1 content and enzyme activity[26]. Our data confirmed these results showed a dose-dependent relationship between CYP 2E1 enzyme activity in human hepatocytes and ethanol exposure.

Lipid peroxidation (and associated membrane damage) is a key feature in alcoholic liver injury. It could result in increased excretion of malondialdehyde in urine of rats following a short- and long-term administration of relatively low doses of ethanol[27]. Lipid peroxidation could result not only from the increased oxygen radical production by the induction of CYP 2E1, but also from the enhanced generation of acetaldehyde shown to cause lipid peroxidation in addition to the hepatic depletion of natural antioxidants such as carotenoids and tocopherol[28]. In the present work, we demonstrated that increased MDA levels were directly linked to the presence of ethanol and might lead to massive changes in cellular GSH levels.

GSH, the most abundant antioxidant in cells, plays a major role in the defense against oxidative stress-induced cellular injury and is essential for the maintenance of intracellular redox balance. In the liver, cellular GSH is determined by a balance between the rate of its synthesis and its utilization and loss. Endogenous hydrogen peroxide is removed by GSH in the presence of glutathione peroxide and could also be removed by catalyzing peroxisomes[29]. Our data showed that GSH levels in human hepatocytes decreased in the presence of ethanol. A time-dependent GSH depletion was observed when cells were incubated with ethanol 6 h after exposure. This time difference was probably linked to the high basal cellular GSH levels. Therefore, we conclude that human hepatocytes incubated with ethanol can result in an imbalance of the anti-oxidative system, thus leading to ethanol-induced cellular injury.

Heme Oxygenase (HO), a rate-limiting enzyme of bilirubin biosynthesis, is composed of three isoenzymes, HO-1, HO-2 and HO-3. HO-1 activity could be highly expressed in the spleen and liver, and enhanced by oxidative stress, heavy metals or hormones[30]. HO is an enzyme that decomposes intracellular toxic heme into biliverdin via oxygenation. Biliverdin is then reduced by biliverdin reductase, resulting in the production of bilirubin, which has been demonstrated to have a potent anti-oxidative activity. Carbon monoxide (CO) is also produced during biliverdin production and has an additional positive effect on vessels during inflammatory events.

Growing evidence supported a role of HO-1 in protecting cells from oxidative stress[31]. The induction of HO-1 has been implicated as an anti-oxidative defense mechanism. This protection by HO-1 seems to be related not only by the reduction of toxic heme concentrations but also to the production of biliverdin and bilirubin, which have been shown to be potent antioxidants. The degradation of heme could also lead to the production of CO, which has been suggested to function as a signaling molecule similar to nitric oxide. With

regard to the liver, CO is involved in the regulation of hepatobiliary functions as cytochrome P450-dependent biotransformation, and in addition, HO-1-derived CO has been shown to protect the hepatic microcirculation under stress conditions. Moreover, enzymatic degradation of heme by HO produces iron. Iron is an essential cofactor of numerous cellular enzymes and redox-dependent proteins, although excess iron has been shown to be cytotoxic via the production of ROI by the Fenton chemistry[32]. More recently it has been demonstrated that cytoprotection of cell cultures by HO-1 may be attributed to augmented cellular iron efflux[33].

Our experiments showed that pre-incubation of CoPP protected human hepatocytes from ethanol-induced cellular injury. The positive HO-1 effect was seen by reducing cellular MDA and increasing cellular GSH levels. The HO-1 induced positive effect was partially reversed by ZnPP. It is interesting to note that simultaneous incubation of CoPP with ethanol did not show any protection. However, we got our results only from human hepatocytes. If they could be proved in human and animals, then they will provide a possible tool to develop new strategies to overcome ethanol abuse in humans. More research work need to be done in the future.

REFERENCES

1. Thurman, D. J., Alverson, C., Dunn, K. A., Guerrero, J., and Sniezek, J. E. (1999). Traumatic brain injury in the United States: A public health perspective. *J. Head Trauma Rehabil.* **14**, 602-615.
2. Henderson, G. I., Chen, J. J., and Schenker, S. (1999). Ethanol, oxidative stress, reactive aldehydes, and the fetus. *Front Biosci*. **4**, D541-550.
3. Cook, M. N., Marks, G. S., Vreman, H. J., McLaughlin, B. E., Nakatsu, K., Stevenson, D. K., and Brien, J. F. (1997). Carbon monoxide formation in the guinea pig hippocampus: ontogeny and effect of *in vitro* ethanol exposure. *Brain Res. Dev. Brain Res*. **101**, 283-286.
4. McCoubrey, W. K., Huang, T. J., and Maines, M. D. (1997). Heme oxygenase-2 is a hemoprotein and binds heme through heme regulatory motifs that are not involved in heme catalysis. *J. Biol. Chem*. **272**, 12568-12574.
5. McCoubrey, W. K., Huang, T. J., and Maines, M. D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur. J. Biochem*. **247**, 725-732.
6. Otterbein, L. E., Lee, P. J., Chin, B. Y., Petrache, I., Camhi, S. L., Alam, J., and Choi, A. M. (1999). Protective effects of heme oxygenase-1 in acute lung injury. *Chest* **116**, 61S-63S.
7. Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., and Koizumi, S. (1999). Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J. Clin. Invest* **103**, 129-135.
8. Immenschuh, S. and Ramadori, G. (2000). Gene regulation of heme oxygenase-1 as a therapeutic target *Biochem. Pharmacol*. **60**, 1121-1128.
9. Otterbein, L. E. and Choi, A. M. (2000). Heme oxygenase. colors of defense against cellular stress. *Am. J. Physiol. Lung Cell Mol. Physiol*. **279**, L1029-1037.
10. Vachharajani, T. J., Work, J., Issekutz, A. C., and Granger, D. N. (2000). Heme oxygenase modulates selectin expression in different regional vascular beds. *Am. J. Physiol. Heart Circ. Physiol*. **278**, H1613-1617.
11. Buzaleh, A. M., Vazquez, E. S., and Batlle, A. M. (1995). Alterations in fluorinated ether anesthetics effects on heme metabolism following chronic ethanol consumption. *Gen. Pharmacol.* **26**, 1425-1429.
12. Okuno, F., Arai, M., Sujita, K., Eto, S., and Ishii, H. (1991). Alterations in hepatic delta-aminolevulinic acid synthetase and heme oxygenase activities after chronic ethanol consumption in rats. *Alcohol*. **8**, 449-451.
13. Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem*. **162**, 156-159.
14. Yoshida, T., Biro, P., Cohen, T., Muller, R. M., and Shibahara, S. (1988). Human heme oxygenase cDNA and induction of its mRNA by hemin. *Eur. J. Biochem*. **171**, 457-461.
15. Du, Breuil, R. M., Patel, J. M., and Mendelow, B. V. (1993). Quantitation of b-actin-specific mRNA transcripts using xeno-competitive PCR. PCR. *Methods Appl*. **6**, 57-59.
16. Shu, Z., Jung, M., Beger, H. G., Marzinzig, M., Han, F., Butzer, U., Bruckner, U. B., and Nussler, A. K. (1997). pH-dependent changes of nitric oxide, peroxynitrite, and reactive oxygen species in hepatocellular damage. *Am. J. Physiol*. **273**, G1118-1126.
17. Jung, M., Drapier, J. C., Weidenbach, H., Renia, L., Oliveira, L., Wang, A., Beger, H. G., and Nussler, A. K. (2000). Effects of hepatocellular iron imbalance on nitric oxide and reactive oxygen intermediates production in

带格式的：项目符号和编号

a model of sepsis. *J. Hepatol*. **33**, 387-394.
18. Woo, J., Iyer, S., Cornejo, M. C., Mori, N., Gao, L., Sipos, I., Maines, M., and Buelow, R. (1998). Stress protein-induced immunosuppression: inhibition of cellular immune effector functions following overexpression of haemoxygenase (HSP 32). *Transpl. Immunol*. **6**, 84-93.
19. Akerboom, T. P. and Sies, H. (1981). Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol.* **77**, 373-382.
20. Wright, J. R., Colby, H. D., and Miles, P. R. (1981). Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. *Arch. Biochem. Biophys*. **206**, 296-304.
21. Zanelli, U., Longo, V., Paolicchi, A., and Gervasi, P. G. (2000). Stabilization of cytochrome P4502E1 protein by ethanol in primary hamster hepatocyte cultures. *Toxicol. In Vitro* **14**, 69-77.
22. Goasduff, T. and Cederbaum, A. I. (2000). CYP2E1 degradation by *in vitro* reconstituted systems: role of the molecular chaperone hsp90. *Arch. Biochem. Biophys*. **379**, 321-330.
23. Cook, M. N., Marks, G. S., Vreman, H. J., Nakatsu, K., Stevenson, D. K., and Brien, J. F. (1997). Heme oxygenase activity and acute and chronic ethanol exposure in the hippocampus, frontal cerebral cortex, and cerebellum of the near-term fetal guinea pig. *Alcohol*. **14**, 117-124.
24. Chen, Q., Galleano, M., and Cederbaum, A. I. (1998). Cytotoxicity and apoptosis produced by arachidonic acid in HepG2 cells overexpressing human cytochrome P-4502E1. *Alcohol. Clin. Exp. Res.* **22**, 782-784.
25. Ponsoda, X., Bort, R., Jover, R., Gomez-Lechon, M. J., and Castell, J. V. (1999). Increased toxicity of cocaine on human hepatocytes induced by ethanol: role of GSH. *Biochem. Pharmacol*. **58**, 1579-1585.
26. Carroccio, A., Wu, D., and Cederbaum, A. I. (1994). Ethanol increases content and activity of human cytochrome P4502E1 in a transduced HepG2 cell line. *Biochem. Biophys. Res. Commun*. **203**, 727-733.
27. Choi, A. M. and Alam, J. (1996). Heme oxygenase-1 function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Respir. Cell Mol. Bio*. **115**, 9-19.
28. Leo, M. A., Rosman, A. S., and Lieber, C. S. (1993). Differential depletion of carotenoids and tocopherol in liver disease. *Hepatology* **17**, 977-986.
29. Kaplowitz, N., Aw, T. Y., and Ookhtens, M. (1985). The regulation of hepatic glutathione. *Annu. Rev. Pharmacol. Toxicol*. **25**, 715-744.
30. Raju, V. S., McCoubrey, W. K., and Maines, M. D. (1997). Regulation of heme oxygenase-2 by glucocorticoids in neonatal rat brain: characterization of a functional glucocorticoid response element. *Biochim. Biophys. Acta* **1351**, 89-104.
31. Liang, M., Croatt, A. J., and Nath, K. A. (2000). Mechanisms underlying induction of heme oxygenase-1 by nitric oxide in renal tubular epithelial cells. *Am. J. Physiol. Renal Physiol*. **279**, 728-735.
32. Suematsu, M., Goda, N., Sano, T., Kashiwagi, S., Egawa, T., Shinoda, Y., and Ishimura, Y. (2000). Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J. Clin. Invest*. **96**, 2431-2437.
33. Ferris, C. D., Jaffrey, S. R., Sawa, A., Takahashi, M., Brady, S. D., Barrow, R. K., Tysoe, S. A., Wolosker, H., Baranano, D. E., Dore, S., Poss, K. D., and Snyder, S. H. (1999). Heme oxygenase-1 prevents cell death by regulating cellular iron. *Nat. Cell Biol.* **1**, 152-157.

(*Received August* 1, 2003 *Accepted April* 8, 2004)