The protective role of HO-1 and its generated products (CO, bilirubin, and Fe) in ethanol-induced human hepatocyte damage

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Yao P, Hao L, Nussler N, Lehmann A, Song F, Zhao J, Neuhaus P, Liu L, Nussler A. The protective role of HO-1 and its generated products (CO, bilirubin, and Fe) in ethanol-induced human hepatocyte damage. Am J Physiol Gastrointest Liver Physiol 296: G1318–G1323, 2009. First published March 26, 2009; doi:10.1152/ajpgi.00555.2007.—It has been reported that naturally occurring quercetin exerts protective effects through heme oxygenase-1 (HO-1) induction. However, the precise mechanism of how ethanol-associated liver damage is countered by quercetin-enhanced HO-1 metabolism still remains unclear. To further decipher the protective role of quercetin on ethanol-induced liver damage, we treated human hepatocytes with quercetin and various (end) products of the HO-1 pathway. Our data clearly showed that quercetin treatment attenuated ethanol-induced damage, whereas hemoglobin and zinc protoporphyrin 9 (ZnPP) abolished such effects. Iron-II aggravated ethanol toxicity and was only partially reduced by quercetin. In contrast, carbon monoxide (CO) dose dependently inhibited ethanol-induced cytochrome P450 2E1 (CYP 2E1) activity and hepatotoxicity but had no influence on CYP 2E1 protein expression. Similarly, hemoglobin dramatically stimulated CYP 2E1 activity but not the protein expression in quercetin- and ethanol-cotreated hepatocytes. ZnPP significantly promoted CYP 2E1 protein expression in the presence and absence of CO treatment but inhibited ethanol-induced CYP 2E1 activation following CO incubation in quercetin- and ethanol-cotreated hepatocytes. These results suggested that quercetin virtually attenuated ethanol-derived oxidative damage via HO-1 induction. Heme degradation and CO release may mediate the protective effects through inhibiting ethanol-induced CYP 2E1 synthesis and enzymatic activity, respectively.

quercetin; CYP 2E1; heme oxygenase-1; carbon monoxide

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2E1 overactivation, is thought to reflect the potential of hepatocytes from ethanol-induced damage (2, 4, 6).

After decades of quiescence, microsomal heme oxygenase (HO), including its inducible isoform HO-1, has recently attracted particular interest because of its extensive physiological modulating functions (25, 30, 34). HO-1 induction by various stresses or selective overexpression represents a crucially cytoprotective pathway both in vitro and in vivo (12, 17, 30). Our earlier researches showed that HO-1 induction by heme and quercetin protects human hepatocytes from ethanol-induced oxidative damage (40). Furthermore, the phenotype of the HO-1 knockout mouse or HO-1 deficiency human, characterized by increased sensitivity to oxidative stress and chronic hepatic inflammation, highlights the biological significance of HO-1 (18, 28). With respect to the potential toxicity of conventional HO-1 inducers, including hemin (31) and cobalt protoporphyrin (CoPP) (13), naturally occurring quercetin, a ubiquitous bioflavonoid found in many plant-derived foods, has gained great interest in preventing oxidative stress-associated diseases by inducing HO-1 (2, 40).

Not surprising is that the physiological function of HO-1 is consequently involved in its catalysis on specific metabolic reaction. HO-1 is the rate-limiting enzyme decomposing heme into biliverdin, free iron, and carbon monoxide (CO) by adding an oxygen molecule to the porphyrin ring of heme. Biliverdin is subsequently metabolized into bilirubin by biliverdin reductase (17, 30). HO-1 metabolism was, however, viewed as a potentially toxicological pathway for many years because of its destruction on heme-containing proteins (hemoproteins) (20, 31) and various “waste” products (23, 30). Interestingly, the prominent antioxidant, anti-inflammatory, and antiapoptotic effects of the physiological concentrations of CO, biliverdin, and bilirubin have been collectively reported on during the past decade (17, 30, 37). Moreover, free heme with excessive amounts greatly increases cellular susceptibility to oxidative stress by acting as a potent hydrophobic prooxidant (20, 31). These studies strongly suggest the hypothesis that HO-1 induction by quercetin may enhance the resistance to ethanol hepatotoxicity by restricting heme-containing CYP 2E1 synthesis and CYP 2E1-dependent oxidative stress induced by ethanol. In the present study, we therefore explore the pharmacological consequences of HO-1 induction through quercetin and in particular how its catabolites (CO, bilirubin, and Fe) protect against ALD in human hepatocytes.
MATERIALS AND METHODS

Materials. Williams’ E medium (with l-glutamine), HEPES buffer, calf serum, sodium pyruvate, penicillin, and streptomycin were obtained from Invitrogen Life Technologies (Scotland, UK). Collagenase P (from clostridium histolyticum) was purchased from Roche Diagnostics (Mannheim, Germany). Hydrocortisone and human insulin were purchased from Merck (Darmstadt, Germany) and Aventis Pharma (Bad Soden, Germany), respectively. Western blotting detecting reagents (enhanced chemiluminescence) and anti-mouse and anti-rabbit secondary IgG were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Reblot buffer was supplied by Chemicon (Temecula, CA). Rabbit polyclonal antibody CYP 2E1 was provided by Calbiochem-Novabiochem (La Jolla, CA). The lactate dehydrogenase (LDH) and aspartate transaminase (AST) essay kits were supplied by Hitado Diagnostic Systems (Möhnesee, Germany). All other reagents were obtained from Sigma (St. Louis, MO).

Isolation and culture of human hepatocytes. Normal human liver tissue samples were obtained from tissue resections of tumor patients with primary and secondary tumors. The study was approved by the ethics committee of the Charité, University Medicine Berlin, and all patients gave their written, informed consent prior to enrollment in the study. The study adhered to the principles of the Declaration of Helsinki as well as to Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001.

Human hepatocytes were isolated by a two-step collagenase perfusion technique followed by a Percoll gradient centrifuge for purification, as previously described (26). Hepatocyte viability assessed by light microscopy was according to the trypan blue exclusion test consistently above 90%. The freshly harvested human hepatocytes were cultured onto rat-tail collagen-coated six-well plates or 90-mm Petri dishes with 95% air and 5% CO2 at 37°C. The medium consisted of Williams’ E medium supplemented with 10% calf serum, 1 M insulin, 15 mM HEPES, 1.4 M hydrocortisone, 100 U/ml penicillin, and 100 mg/ml streptomycin. The following day, the hepatocytes were incubated for 24 h with medium containing various pharmacological reagents, and then the cells and supernatant were collected for various biosassays according to the corresponding experimental protocol.

Activity assay of enzymatic LDH, AST, and CYP 2E1. AST levels in culture medium were measured by the enzymatic kinetic method using the commercially available reagent kits. Enzymatic activity of microsomal CYP 2E1 was tested according to hydroxylation rate of p-nitrophenol into 4-nitrocatechol (7). Hepatocytes were incubated with 0.5 mM p-nitrophenol for 60 min and then stopped by 5% trichloracetic acid. The absorbance was recorded following NaOH treatment, and the activity was calculated using the extinction coefficient: 10.28 M/Mcm/cm^{-1}.

Measurement of hepatic malondialdehyde. Measurement of hepatic malondialdehyde (MDA) and glutathione (GSH) content MDA, one of the main oxidative degradation products of lipid peroxidation, was estimated by determining the production of thiobarbituric acid reactive components with 1,1,3,3-tetraethoxypropane as an external standard (38). The measurement of cellular reduced GSH, a major form of the main oxidative degradation products of lipid peroxidation, was estimated by the basis of its reaction with 5,5’-dithiobis-2-nitrobenzoic acid to generate 2-nitro-5-thiobenzoic acid following deproteinization by 5% trichloracetic acid (24).

Western blot analysis for CYP 2E1 expression. Microsomal protein (30 µg) was separated by 12.5% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane (Bio-Rad, Munich, Germany). The membranes were blocked with 5% nonfat milk solution overnight and incubated with rabbit CYP 2E1 polyclonal antibody (1:1,000) for 1.5 h, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2,000) for 1.5 h. After being washed, the immune complex on the membrane was developed using a chemiluminescence detection system. Reference β-actin was estimated by stripping the membrane with a reblot buffer and reincubating with corresponding antibody.

Statistical analysis. Data were expressed as means ± SD from triplicates of at least three independent hepatocyte isolations and subjected to one-way ANOVA followed by Student-Newman-Keuls multiple range test (SPSS 12.0 software package). Statistical significance was established at a P < 0.05.

RESULTS

Effect of metabolites originated from HO-1 induction on ethanol-induced oxidative damage in human hepatocytes. Compared with untreated human hepatocytes, ethanol exposure (100 mM, 24 h) of human hepatocytes resulted in a dramatic leakage of AST and LDH to the culture medium and a sustained cellular GSH depletion, accompanying a parallel elevation of MDA equivalence. In contrast, quercetin substantially reversed such detrimental effects resulting from ethanol exposure (Figs. 1 and 2). Our previous research showed that HO-1 induction by quercetin contributed to the cytoprotection against ethanol-derived oxidative damage (40). To explore which precise metabolite(s) of heme degradation mediate the protective effects, we evaluated the cellular damage of ethanol-incubated hepatocytes in the presence of various exogenous reagents or antagonists involved in the HO-1 pathway. As shown in Figs. 1 and 2, hemoglobin (100 µM), a potent scavenger of CO, completely abolished quercetin-elicited cytoprotection and aggravated ethanol-induced hepatotoxicity compared with data generated from ethanol plus quercetin. Exogenous added bilirubin (10 µM) failed to inhibit ethanol-induced GSH depletion as well as leakage of AST and LDH (Figs. 1A and 2B). Bilirubin or hemoglobin alone did not show any toxicity in the used concentration (data not shown); however, the addition of ferrous sulfate itself (10 µM) increased the toxicity toward hepatocytes dramatically and also increased the oxidative cytotoxicity of ethanol by 60%. Deferoxamine (100 µM), a chelator of free iron, exhibited no additional protective effects when incubated with quercetin on ethanol-induced cytoxicity (Figs. 1B and 2B). Incubation of human hepatocytes with iron plus ethanol resulted in an almost twofold increase of AST, which, however, was only partially reduced by the addition of quercetin, demonstrating a protective effect of quercetin (Fig. 1B). In the same line of evidence, we found that quercetin only partially reversed the adverse effects of a combination of ethanol and iron on cellular GSH and MDA levels (Fig. 2B).

Effect of CO on ethanol-induced oxidative damage in human hepatocytes. Physiological concentration of CO is mainly derived from intrinsic heme degradation by HO isoforms, especially by HO-1 under stress conditions (30). Ruthenium-containing CO-releasing compound (tricarbonyldichlororuthenium dimmer, CORM-2) has been confirmed to possess similar effects as a gas as CO administration, which provides a useful tool to study the mechanism of CO action (33). As shown in Fig. 3 and Fig. 4, CORM reduced the release of cellular AST and LDH induced by ethanol in a dose-dependent manner (within 20 µM). Furthermore, we observed a parallel inhibition of MDA elevation and GSH depletion following CORM treatment for ethanol-incubated hepatocytes. In contrast, the inactive form of CORM (iCORM) did not show any beneficial effect on ethanol-elicited oxidative damage, and CORM itself...
had no influence on any such measured parameters. It is noteworthy that 50 μM CORM consistently exhibited a weaker cytoprotection than at 20 μM (data not shown).

**Effect of HO-1/CO on the expression and activity of CYP 2E1.** CYP 2E1 is an important cytochrome responsible for ethanol metabolism and hepatotoxicity (4). With respect to the extensive affinity of CO on various heme-containing molecules, including CYP 2E1, we here further examined the potential influence of CO or HO-1 induction on CYP 2E1 expression and activity. As expected, ethanol virtually triggered the protein expression and enzymatic activity of CYP 2E1. The inhibition of CYP 2E1 by classic diallyl disulfide (50 μM) partially protected hepatocytes from ethanol-induced oxidative stress. Notably, the activity of CYP 2E1 was dose dependently inhibited, but the protein expression was kept unchanged following CORM treatment in ethanol-treated hepatocytes compared with untreated control cultures (Figs. 3, 5 and 6). Quercetin substantially inhibited both the expression and activity of CYP 2E1 in ethanol-exposed hepatocytes compared with ethanol-treated cells alone, which is similar to the effect of hemin (20 μM), a typical inducer of HO-1. However, quercetin itself seemed to have no inhibitive effect on the constitutive expression or activity of CYP 2E1 in untreated cells. Hemoglobin abrogated the quercetin-derived inhibitive effect and further stimulated ethanol-induced CYP 2E1 activation, underlying the central role of CO in CYP 2E1 inhibition. Interestingly, hemoglobin had no impact on the protein expression of CYP 2E1 compared with the findings of hepatocytes coincubated with quercetin plus ethanol (Figs. 5 and 6).

Considering the inductive effect of ethanol and quercetin on HO-1 (40) and their potential influence on heme-containing CYP 2E1 synthesis attributable to their heme substrate catabolism, we adopted zinc protoporphyrin 9 (ZnPP, 25 μM) to block HO bioactivity and accordingly cancelled the effect of endogenous CO from the HO system in ethanol- and/or quercetin-incubated hepatocytes. A higher activity and expression of CYP 2E1 was observed in hepatocytes treated with ZnPP.
plus quercetin in the presence of ethanol than in cells incubated with ethanol or quercetin plus ethanol alone. These results suggested that ZnPP abolished the inhibiting effect of quercetin and significantly promoted the induction of CYP 2E1 by ethanol on both enzymatic activity and protein expression profiles (Figs. 5 and 6). Additionally, we found that ZnPP treatment led to an increased CYP 2E1 activity in hepatocytes coincubated with ethanol plus CORM, indicating that ZnPP decreased the inhibitive effect of CORM on CYP 2E1 activity (Figs. 3 and 5). Addition of CORM to hepatocytes incubated with a mixture of ZnPP, quercetin, and ethanol did not result in changes of CYP2E1 protein expression but in reduced CYP 2E1 enzymatic activity. In contrast to CORM, iCORM had no influence on both profiles of CYP 2E1 (Figs. 5 and 6).

**DISCUSSION**

Increasing evidence supports that HO-1 may function as a special “therapeutic funnel” against stress-associated physiological disorders on the basis of its rapid upregulation under various stress conditions and potent physiological regulating properties. Therefore, HO-1 induction has been suggested to have a general adaptive response and enhanced resistance to various stresses (30, 35, 37). Accordingly, pharmacological activation of HO-1 is a novel therapeutic intervention for various diseases involved in oxidative stress (2, 3). In our present and previous studies (40), HO-1 induction by quercetin or hemin evidently reduced ethanol-induced hepatotoxicity and redox disorders, an effect abolished by ZnPP. Similar beneficial effects of HO-1 induction against ethanol toxicity have been reported on hippocampal neurons, gastric mucosa, and hepatic tissue (10, 15, 19, 41). More importantly, our results may provide a promising pharmacological application of nat-

![Fig. 3](image3.png)

**Fig. 3.** Dose response of CO on CYP 2E1 activity and leakage of AST and LDH of ethanol-treated hepatocytes. Human hepatocytes were incubated with ethanol, tricarbonyldichlororuthenium dimmer (CORM)/inactive CORM (iCORM) or diallyl disulfide (DADS) for 24 h. CORM was freshly dissolved in DMSO; iCORM was a result of storing the CORM solution at room temperature for 24 h. The activity of CYP 2E1, AST, and LDH was measured as described in MATERIALS AND METHODS. Data were expressed as means ± SD from triplicates of at least 3 independent hepatocyte isolations. ΔP < 0.05 vs. untreated hepatocytes; *P < 0.05 vs. ethanol-treated hepatocytes.

![Fig. 4](image4.png)

**Fig. 4.** Dose response of CO on GSH and MDA content of ethanol-treated hepatocytes. Human hepatocytes were incubated with ethanol, CORM/iCORM, or DADS for 24 h and then harvested for GSH and MDA assay by colorimetric method. Data were expressed as means ± SD from triplicates of at least 3 independent hepatocyte isolations. ΔP < 0.05 vs. untreated hepatocytes; *P < 0.05 vs. ethanol-treated hepatocytes.

![Fig. 5](image5.png)

**Fig. 5.** Influence of HO-1 induction and CO releasing by quercetin on CYP 2E1 activity of ethanol-treated hepatocytes. Human hepatocytes were incubated with ethanol, quercetin, and/or other reagents for 24 h. CYP 2E1 activity was measured by hydroxylation rate of p-nitrophenol. Data were expressed as means ± SD from triplicates of at least 3 independent hepatocyte isolations. H, hemin (20 μM); ZP: zinc protoporphyrin 9 (ZnPP, 25 μM); CO, CORM (20 μM, prepared freshly); iCO, iCORM (20 μM, prepared by leaving CORM solution at room temperature for 24 h). ΔP < 0.05 vs. untreated hepatocytes; *P < 0.05 vs. ethanol-treated alone hepatocytes.

![Fig. 6](image6.png)

**Fig. 6.** Effect of HO-1 induction and CO release by quercetin and its outcome on CYP 2E1 expression of ethanol-treated hepatocytes. Human hepatocytes were incubated with ethanol, quercetin, and/or other reagents for 24 h. CYP 2E1 expression was estimated by Western blot assay. The results were expressed as relative ratio of Nrf2 against β-actin (means ± SD) from 3 independent hepatocyte isolations. ΔP < 0.05 vs. untreated hepatocytes; *P < 0.05 vs. ethanol-treated alone hepatocytes; #P < 0.05 vs. hepatocytes cotreated with quercetin and ethanol.
urally occurring quercetin by functioning as a novel nontoxic HO-1 inducer, different from the typical inducers that have potential prooxidant toxicity (13, 31). The prospect that quercetin antagonizes oxidative stress through HO-1 pathway is further supported by studies performed in murine RAW264.7 macrophage cell line and aortic smooth muscle cells (5, 21).

Given the potential physiological importance of HO-1 in mediating cellular homeostasis as a general inducible stress protein response, a special effort has been focused on characterizing the functional role of heme catabolites. Once viewed as waste or toxic product, bilirubin is now suggested to display a beneficial role in a number of diseases, including atherosclerosis, inflammatory, autoimmune, and degenerative diseases, and cancer by serving as a highly lipophilic antioxidant (17, 36). In the present study, bilirubin slightly lowered ethanol-induced lipid peroxidation as reflected by the decreased MDA content but failed to inhibit GSH depletion and membrane damage, indicating limited cytoprotection of bilirubin against ethanol-induced hepatotoxicity, which is in agreement with earlier work (14). However, contradictory results have been reported that bilirubin virtually mediated the HO-1 defense from ethanol-induced neurotoxicity (19) and hemin-elicted prooxidative toxicity (11). In addition, our data showed that exogenous iron itself was critically toxic to hepatocytes and aggravated ethanol-induced hepatotoxicity but was partially corrected by the addition of quercetin. Deferoxamine, however, had no synergetic protective effects on quercetin. It is well known that free iron plays a critical catalytic role in the multiplication of chain reactions of free radical formation, NO-dependent thiols nitrosylation, and Haber-Weiss reaction, under which the progression of lipid peroxidation is significantly accelerated (30). Therefore, our results suggest that the iron release from the HO-1 system had no or little influence on cellular iron homeostasis. The parallel induction of ferritin through HO-1 may serve as a safe iron repository to maintain homeostasis through prompt sequestration of unbound iron with remarkable capacity (30).

Despite its potential toxicity, gaseous CO has still received considerable attention as a signaling molecule with vasodilatory effects mediated via cGMP, as well as antiapoptotic and anti-inflammatory effects (14, 30). Analogous to NO, CO can freely travel throughout intracellular and extracellular compartments and exert a wide spectrum of physiological modulating effects on multisystems (8). In the present study, we found that the protective effect of HO-1 induction by quercetin against ethanol-induced hepatotoxicity was completely inhibited by the addition of hemoglobin, or CO in a dose-dependent manner. These results suggest that CO could mediate the defensive effect among HO-1 metabolites through inactivating CYP 2E1 enzyme activity. This is supported by observations showing that CYP 2E1 transfection upregulates HO-1 expression and CO production, whereas bilirubin has no impact on CYP2E1-dependent arachidonic acid toxicity (14). Similar observations have been reported in experimental cerebral malaria (27) and transplant-associated ischemia-reperfusion cardiac injury (1). High affinity of CO for heme moieties strongly suggests that heme-containing enzymes, such as cytochrome P450, cyclooxygenase, guanylyl cyclase, and NO synthase (NOS), are possible targets for CO-dependent cellular activities (20, 22, 33). CYP 2E1 is a typical hemoprotein responsible for ethanol-associated oxidative stress, and, hence, its inactivation is attributable to CO binding, which might be one of the crucial mechanisms by which HO-1 induction (by quercetin) attenuates ethanol-dependent hepatotoxicity.

Undoubtedly, the HO-1 substrate heme is essential for the synthesis of various hemoproteins, possessing important physiological functions by which heme virtually acts as a signaling molecule that controls diverse cellular processes ranging from signal transduction to protein complex assembly (23). These hemoproteins presumably undergo a rapid turnover during oxidative stress (9), whereas HO-1 induction may restrict their synthesis because of heme decomposition (14, 22). In the present study, we found that ethanol stimulated CYP 2E1 expression and that it is downregulated in the presence of HO-1 induction by quercetin or hemin. ZnPP treatment not only removed the inhibitive effect of quercetin but also remarkably upregulated ethanol-induced CYP 2E1 expression. Hemoglobin or CORM showed no influence on CYP 2E1 expression under this condition, indicating that HO-1 induction, but not the CO release, downregulates ethanol-dependent CYP 2E1 expression. Similar observations have been reported on cyclooxygenase (22) and inducible NOS (16, 32). Thus heme degradation catalyzed by HO-1 may provide an alternative haptoprotective mechanism of quercetin by limiting the ethanol-induced CYP 2E1 synthesis.

Taken together, naturally occurring quercetin evidently attenuates ethanol-derived oxidative damage via HO-1 induction. Heme cleavage and CO release may contribute to the protective effect through inhibiting ethanol-induced CYP 2E1 synthesis and enzymatic activity, respectively. In our experiments, the catabolites iron and bilirubin of the HO-1 pathway had no protective role underlying a particular role for CO in HO-1 induction. Considering the potential toxicity of hemin or CoPP as a classic HO-1 inducer, the present study may highlight the promising prospect to prevent ALD and other oxidative stress-related disorders by quercetin or other natural occurring bioflavonoids.

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Hepatocyte Protection via HO-1/CO