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

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1Universitätsmedizin Berlin, Charité, Campus Virchow, Department of General, Visceral, and Transplantation Surgery, Berlin, Germany; 2Tongji Medical College, Huazhong University of Science and Technology, School of Public Health, Department of Nutrition and Food Hygiene, Wuhan, People’s Republic of China; 3Technical University Munich, Department of Traumatology, Munich, Germany

Submitted 28 November 2007; accepted in final form 23 March 2009

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 hepatoprotective effects through heme oxygenase-1 (HO-1) induction. However, the precise mechanism of how ethanol-associated liver damage is counteracted 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 ethanolderived 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

Alcohol abuse is one of the major public health problems in the world because of its extensive detrimental effects on the alimentary, circulatory, immune, and nervous systems (29, 39). The most extensively investigated health risk of ethanol is alcoholic liver disease (ALD), characterized by a spectrum of liver damages ranging from steatosis and hepatitis to cirrhosis, and in some cases even leading to hepatocellular carcinoma (6). Oxidative damage to macromolecules during ethanol metabolism plays a critical role in the progression of ALD. Induction of cytochrome P450 2E1 (CYP 2E1) by ethanol is one of the central pathways by which ethanol generates a state of oxidative stress in hepatocytes. Thus the counteraction capacity against ethanol-induced redox disorders, especially originated from CYP 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 hemin 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) assay kits were supplied by Hitato Diagnostic Systems (Mühlheim, 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 assayed 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 bioassays 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% trichloroacetic acid. The absorbance was recorded following NaOH treatment, and the activity was calculated using the extinction coefficient: 10.28 mM/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 reacting with 5,5′-dimethythiobis-2-nitrobenzoic acid to generate 2-nitro-5-thiobenzoic acid following deproteinization by 5% trichloroacetic 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-elicted 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 cytotoxicity (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-elicted 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, underlining 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 coinubated 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

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**Fig. 1.** A: effect of quercetin-derived heme oxygenase-1 (HO-1) catabolites on aspartate transaminase (AST) and lactate dehydrogenase (LDH) leakage from ethanol-incubated hepatocytes. Human hepatocytes were incubated with ethanol, quercetin, and/or other reagents for 24 h. Supernatant was collected from AST and LDH by using commercially available reagent kits. Data were expressed as means ± SD from triplicates of at least 3 independent hepatocyte isolations. N, normal control; E, ethanol (100 mM); Q, quercetin (100 μM); Hb, hemoglobin (100 μM); B, bilirubin (10 μM); CYP 2E1, cytochrome P450 2E1. *P < 0.05 vs. untreated hepatocytes; *P < 0.05 vs. ethanol-treated hepatocytes alone; #P < 0.05 vs. hepatocytes cotreated with quercetin and ethanol. B: effect of iron supplementation in the presence of ethanol and quercetin on the cellular AST release. Fe, ferrous sulfate (10 μM); Df, defereroxamine (100 μM). *P < 0.05 vs. ethanol plus iron-treated hepatocytes alone.

**Fig. 2.** A: impact of quercetin-derived HO-1 catabolites on glutathione (GSH) and malondialdehyde (MDA) content in ethanol-treated hepatocytes. Human hepatocytes were incubated with ethanol, quercetin, and/or other reagents for 24 h, and thereafter GSH and MDA levels were measured using the colorimetric method. Data were expressed as means ± SD from triplicates of at least 3 independent hepatocyte isolations. *P < 0.05 vs. untreated hepatocytes; *P < 0.05 vs. ethanol-treated alone hepatocytes; *P < 0.05 vs. ethanol plus iron-treated hepatocytes alone. **B:** effect of iron supplementation in the presence of ethanol and quercetin on the cellular GSH and MDA content. *P < 0.05 vs. ethanol plus iron-treated hepatocytes alone.
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...
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-elicited induced lipid peroxidation as catalyzed by HO-1 may provide an alternative hepato-protective 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 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.

ACKNOWLEDGMENTS

We gratefully acknowledge Ms. Evelyn Hungerbüher and Ms. Anja Schirmeier for excellent technical assistance and Ms. Sylvia Albrecht for careful editorial work.

GRANTS

This work was supported by the Federal Ministry of Research (BMBF 0313079B, BMBF 01GG0732, BMBF 0315208E) and Fresenius Biotech, Bad Homburg, Germany.

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