Role of heme oxygenase-carbon monoxide pathway in pathogenesis of cirrhotic cardiomyopathy in the rat

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Liu, Hongqun, Daisheng Song, and Samuel S. Lee. Role of heme oxygenase-carbon monoxide pathway in pathogenesis of cirrhotic cardiomyopathy in the rat. Am J Physiol Gastrointest Liver Physiol 280: G68–G74, 2001.—The enzyme heme oxygenase (HO), which exists in inducible (HO-1) and constitutive (HO-2) isoforms, degrades heme to biliverdin and CO. CO depresses cardiac contraction via cGMP. We aimed to clarify a possible role for the HO-CO pathway in the pathogenesis of cirrhotic cardiomyopathy in bile duct-ligated rats. Four weeks after bile duct ligation or sham operation, rat ventricles were examined for HO-1 and HO-2 mRNA by RT-PCR and for protein expression by Western blotting. Total HO enzyme activity and cGMP levels were also measured. The effects of a HO inhibitor, zinc protoporphyrin IX (ZnPP), on ventricular cGMP levels and isolated papillary muscle contractility were studied. We found that HO-1 mRNA transcription and protein expression were significantly augmented in cirrhotic hearts compared with sham-operated controls, whereas there was no difference in HO-2 mRNA or protein levels. Total HO activity and cGMP levels were significantly increased in cirrhotic ventricles vs. controls. In cirrhotic ventricles, treatment with ZnPP significantly decreased cGMP production and improved the blunted papillary muscle contractility, whereas it had no effect on control muscles. CO perfusion inhibited papillary muscle contractility, an effect completely blocked by methylene blue and partially blocked by ZnPP. These results indicate that activation of the HO-CO-cGMP pathway is involved in the pathogenesis of cirrhotic cardiomyopathy.

heat shock protein; cardiac contractility; cirrhosis; guanylyl cyclase; guanosine 3′,5′-cyclic monophosphate

CARDIOVASCULAR ABNORMALITIES in cirrhosis include decreased peripheral vascular resistance, increased basal cardiac output, and impaired cardiac contractile responsiveness to stressful stimuli. The latter phenomenon has been termed “cirrhotic cardiomyopathy” (18). The enzyme heme oxygenase (HO), which exists in inducible (HO-1) and constitutive (HO-2) isoforms, catalyzes the oxidation of heme to biologically active molecules: iron, a gene regulator, biliverdin, an antioxidant, and CO, a heme ligand. CO, like nitric oxide (NO), is a short-lived gas with numerous physiological roles. In recent years, many of the physiological roles of CO have been elucidated in several systems such as the cardiovascular, nervous, and immune systems (reviewed in Ref. 23). In mammalian cells, the only source of CO generation is by the oxidation of the heme molecule by the microsomal HO system (33). The HO-1 gene responds to a huge number of stimuli; indeed, it has proven to be inducible by more categories and types of stimuli than any gene described to date.

CO has been shown to increase cGMP levels by activating guanylyl cyclase (1). cGMP depresses ventricular contractility by inhibiting intracellular calcium fluxes (36). In an experimental canine model of right-sided congestive heart failure, HO-1 gene transcription was increased in the right ventricle, but not the left (30). Suematsu and Ishimura (33) have amply demonstrated an important physiological role of CO as a regulator of sinusoidal vascular tone in the liver. A recent study also showed that in portal hypertensive rats, HO-1 mRNA and protein were expressed in the liver and splanchnic organs (2). However, there has been no study to date of a possible role for CO in the pathogenesis of cardiovascular changes in cirrhosis or portal hypertension. We therefore aimed to clarify whether activation of the HO-CO pathway may play a pathogenic role in the regulation of cardiac contractility in a rat model of cirrhotic cardiomyopathy. This entailed detailed examination of the entire HO-CO pathway, from mRNA of the HO isoforms to protein expression and enzymatic activity of HO, cGMP levels, and testing the effects of a HO inhibitor, zinc protoporphyrin IX (ZnPP), and CO itself on the contractility of cirrhotic papillary muscles. Other compounds with varying degrees of activity on the HO-CO-cGMP pathway such as copper protoporphyrin IX (CuPP) and methylene blue were also evaluated.

METHODS

Reagents and enzymes. DNA synthase, RNA superscript synthase, and PCR buffer were purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). Agarose and ZnPP were obtained from ICN Biomedicals (Aurora, OH). HO-1 and HO-2 antibodies were purchased from Stressgen Biotechnologies (Victoria, BC, Canada), and isoproterenol was purchased from Sigma (St. Louis, MO). Other reagents were purchased from Sigma, Bio-Rad (Hercules, CA), or Fisher Scientific and were the highest available grade.

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Animal model. Male Sprague-Dawley rats between 200 and 250 g, bred locally by the Bioscience Department, University of Calgary, were used in the present study. The protocol was approved by the Faculty of Medicine Animal Experimentation Committee in accordance with guidelines established by the Canadian Council on Animal Care. Cirrhosis was induced according to previously described methods (14, 21). Briefly, rats were anesthetized by halothane inhalation. An abdominal midline incision was used to expose the common bile duct, which was doubly ligated with 4-0 silk suture and cut between the ligatures. The muscle and skin were sutured with 3-0 silk separately. Immediately after surgery, rats were administered 30,000 U penicillin G intramuscularly to prevent infection and allowed to recover in individual cages. Sham-operated control rats received the same laparotomy except that the bile duct was not ligated or sectioned. It was shown previously that 4 wk after ligation, rats demonstrate typical biliary cirrhotic changes in histology (21); accordingly, all experiments were performed 4 wk postoperatively.

To investigate the effect of the HO inhibitor ZnPP on the ventricular cGMP content, a group of bile duct-ligated (BDL) rats (n = 6) and a sham-operated control group (n = 6) were administrated ZnPP (50 μmol/kg body wt ip) 60 min before death. The dose and timing were based on previous studies demonstrating efficacy in inhibiting HO (27). Separate groups of BDL rats (n = 6) and sham-operated controls (n = 6) were also used for ventricular cGMP measurements, but these received no ZnPP treatment.

For the isolated papillary muscle studies, the animals were killed by guillotine decapitation and the muscles were immediately excised and studied. For all other protocols, the animals were killed and the hearts were excised, rinsed in cold diethyl pyrocarbonate-treated water, snap-frozen in liquid nitrogen and stored at −70°C until use.

HO-1 and HO-2 mRNA expression quantified by RT-PCR. Total RNA from the rat ventricular tissue was extracted using the acid guanidinium isothiocyanate method (38). Total RNA was quantified using a GeneQuant spectrophotometer (Pharmacia, Piscataway, NJ). The cDNA was obtained through RT-PCR by the method of Wong et al. (38). Briefly, the reaction mixture containing 2 μg of the total RNA, first-strand buffer, 20 nM each of dATP, dGTP, dCTP, and dTTP, 160 units of Superscript-RT, and 100 pmol of random hexamer oligodeoxynucleotides (Pharmacia) was incubated at 20°C for 10 min. The reverse transcription reaction was carried out in a thermal cycler (Barnstead/Thermolyne, Dubuque, IA) at 42°C for 50 min, and the product was then heated to 95°C to stop the reaction.

HO-1, HO-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; as an internal control) primers were synthesized by Gibco-BRL Life Technologies. Briefly, the initial reaction mixture contained 2 μl of RT product, 1× PCR buffer, 4 nM of each deoxynucleotide, and 2 units of Taq DNA polymerase. For HO-1 and HO-2, the upstream and downstream primers of both were 20 pmol, for the internal control GAPDH, the amount of upstream and downstream primers was 4 pmol. The previously published primer sequences (17, 26) are presented in Table 1. Taq DNA polymerase was added to the PCR reaction mixture in the first denaturation step. A set of 30 cycles was chosen to ensure that the amplification of PCR products was in the exponential range according to preliminary cycle test experiments. In each PCR cycle, heat denaturation was set at 94°C for 1 min, primer annealing at 55°C for 30 s, and polymerization at 72°C for 1 min. PCR product (10 μl) was electrophoresed in 1.5% agarose gels containing 0.2 μg/ml of ethidium bromide. The gels were visualized under ultraviolet light and photographed with Polaroid film. The films were scanned by computerized laser densitometry, and the results are expressed in arbitrary densitometry units normalized to GAPDH expression.

Table 1. HO-1, HO-2, and GAPDH primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequences</th>
<th>Amplicon Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>Upstream: 5′-AGCATGCCCCAGGATTTG-3′</td>
<td>615</td>
</tr>
<tr>
<td></td>
<td>Downstream: 5′-CTAGCTGGATGTTGACGAGGA-3′</td>
<td></td>
</tr>
<tr>
<td>HO-2</td>
<td>Upstream: 5′-GGGACCAAGGAACAGCAT-3′</td>
<td>828</td>
</tr>
<tr>
<td></td>
<td>Downstream: 5′-CTATGTTAGCAGGCGCAAGA-3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Upstream: 5′-CGGAGTCAAGGGTTTGGTCGTAT-3′</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>Downstream: 5′-AGCCTTCCTCCATGTTGAAAGAC-3′</td>
<td></td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, inducible heme oxygenase; HO-2, constitutive heme oxygenase.
NaHCO₃, and 10 glucose at pH 7.4. The muscles were suspended by ligatures in a 15-ml organ bath and connected to an isometric force transducer and recorder (Grass Instruments, Brookline, MA). They were allowed to equilibrate for at least 90 min before any drugs were used. The initial tension was set at 500 mg because previous work showed that at this tension, maximum contractile force can be obtained (19). After equilibration, the muscles were subjected to electrical field stimulation (Grass 88 stimulator) at 1 Hz and 30 V, which is about 20% higher than the threshold. The papillary muscles were then stimulated by increasing isoproterenol doses (10⁻⁸–10⁻⁶ M) to obtain a dose-response curve. For the ZnPPP studies, isoproterenol dose-response curves were constructed 30 min after the papillary muscles were incubated with ZnPPP at a concentration of 10⁻⁶ M. This dose was based on previous studies demonstrating reliable HO activity inhibition at this concentration (32). Additionally, CuPP, which has been shown to have no significant activity on the HO-CO pathway at the same concentration (25), was used as another control in some experiments.

The direct effect of CO on papillary muscle contractility in sham-operated control muscles was also investigated. CO-saturated Tyrode buffer was infused continuously at a rate of 1.5 ml/min in a 10-ml organ bath. This protocol is known to produce a CO concentration of ~1 mM (32). Finally, ZnPPP (10⁻⁶ M) or the guanylyl cyclase inhibitor methylene blue (10⁻⁵ M) was preincubated in the papillary muscle organ bath for 30 min, followed by CO perfusion as above to determine whether CO was acting through guanylyl cyclase activation.

**Statistical analysis.** Results are expressed as means ± SD. For analysis of two independent groups, a Student’s t-test was used. For analysis of three or more independent groups, one-way ANOVA with post hoc Newman-Keuls testing was used to compare group means. The significance level was set at P < 0.05.

**RESULTS**

**Ventricular HO-1 and HO-2 mRNA expression.** A representative HO-1 mRNA RT-PCR Polaroid photograph is presented in Fig. 1. The expression of HO-1 mRNA was barely visible in sham-operated control rats. In contrast, abundant HO-1 mRNA was observed in cirrhotic rats. The pooled data of HO-1 mRNA expression (n = 6 each group) showed significant increases in cirrhotic rats (P < 0.01 compared with sham-operated controls). Western blotting revealed a similar pattern of increased HO-1 content in the cirrhotic hearts (Fig. 2), whereas neither HO-2 mRNA content (Fig. 3) nor HO-2 protein (Fig. 4) differed between BDL and control hearts.

**Ventricular HO activity.** Total HO enzymatic activity was significantly higher in the BDL ventricles than in the sham-operated control group (1,272 ± 150 vs. 264 ± 42 pmol bilirubin·mg protein⁻¹·h⁻¹, respectively; P < 0.01).

**Ventricular cGMP content.** The ventricular concentration of cGMP in cirrhotic rats was significantly higher compared with sham-operated controls, and treatment with the HO inhibitor ZnPPP significantly decreased the cGMP level in the cirrhotic rats (Fig. 5).
In contrast, ZnPP treatment did not significantly affect cGMP levels in the sham-operated control rats.

**Effect of CO and ZnPP on isolated papillary muscle contractility.** As previously demonstrated, baseline isoproterenol-stimulated maximum papillary muscle contractile force was lower in BDL muscles compared with control muscles, confirming the presence of cardiomyopathy (Refs. 18, 21; Fig. 6). When the papillary muscles from cirrhotic rats were preincubated with ZnPP, contractile force increased significantly (Fig. 6). In contrast, ZnPP had no effect on papillary muscle contractility in sham-operated controls. As expected, CuPP had no effect either on BDL or sham-operated control muscles. In sham papillary muscles, CO perfusion directly inhibited contractility (69% of basal contractile force) and ZnPP preincubation partially reversed the CO inhibition (78% of basal contractile force) (Fig. 7). However, preincubation with methylene blue completely abrogated the CO-induced inhibition of contractility (Fig. 7), thus indicating that CO mediates its effects through stimulation of guanylyl cyclase.

**DISCUSSION**

To our knowledge, the current study is the first to examine a possible regulatory role of CO in the cardiovascular system of cirrhosis. We found that HO-1 mRNA transcription and protein expression and total HO activity were significantly upregulated in cirrhotic hearts but not in sham-operated control hearts. In contrast, there were no changes in HO-2 mRNA transcription or protein expression, indicating that the increase in total HO activity is entirely due to HO-1 induction. These results are generally concordant with those of Raju et al. (30), who showed induction of HO-1 but not HO-2 in a canine model of right-sided congestive heart failure.
The issue of CO vs. NO activation is important because recent studies suggested a regulatory role for NO in the genesis of ventricular contractile dysfunction in cirrhosis (3, 37). Indeed, we previously showed induction of NOS 2 (inducible NO synthase) in the heart of BDL rats (16). Because both NO and CO mediate their effects through stimulation of guanylyl cyclase, it was necessary to demonstrate that the increased cardiac cGMP levels we observed were at least partly due to CO rather than NO. Accordingly, the skeptic could argue that the increased gene and protein expression of HO does not affect cardiac function and that the elevated cGMP levels are entirely due to the increased cardiac NOS 2 expression. However, our papillary muscle results with CO and ZnPP effectively refute that suggestion. First, direct exposure of the papillary muscles to a CO-saturated solution produced a significant depression of contractility. Second, ZnPP, which at the concentrations used in our study predominantly inhibits HO activity but also may weakly directly inhibit sGC, partially reversed the CO-induced depression of contractility. Moreover, ZnPP by itself restored the contractility as well as the cGMP levels in cirrhotic ventricles to sham-operated control values. Finally, preincubation with the sGC inhibitor methylene blue completely reversed the negative inotropic effect of CO. Together, these results strongly suggest that CO generated by the action of HO can depress contractility by stimulating sGC to produce cGMP. The methylene blue and ZnPP results indicate that CO mediates its myocardial depressant effect solely through cGMP as a second messenger rather than other possible mechanisms such as lipid peroxidation or through reactive oxygen intermediates.

The reasons for the activation of the HO-CO pathway in cirrhotic cardiomyopathy remain unclear, but we can speculate on several possibilities. In cirrhotic patients and animal models including the BDL rat, the sympathetic nervous system is activated (5, 8), and the resultant increased levels of norepinephrine may stimulate the HO-CO pathway (30). Another possibility to explain the HO-CO pathway activation may be increased cytokinemia in cirrhosis, which has been ascribed to enteric bacterial translocation through the gut wall (4, 35). The resulting increase in portal venous bacteremia and endotoxemia would induce the hepatic and systemic reticuloendothelial cells to produce high cytokine levels. Endotoxin and cytokines could then activate HO-1 mRNA transcription and protein expression (23, 33, 34).

In support of both of the above hypotheses, increased cardiac and serum levels of cytokines such as tumor necrosis factor-α and interleukin-1 (16) and increased serum levels of norepinephrine (5) were reported previously in the BDL rat. However, cirrhosis is associated with abnormalities of numerous hormonal, neural, and local regulatory substances. Given the huge number of diverse stimuli that are capable of inducing HO-1 expression, it is not only possible that several factors are involved but also likely that pinpointing the exact cause(s) will be extremely difficult.
Over the past decade, our laboratory has attempted to clarify the pathogenic factors underlying the depressed ventricular contractile responsiveness in the BDL rat (reviewed in Refs. 15 and 18). To date, we have discovered evidence for 1) downregulation of the stimulatory β-adrenergic signaling pathway, manifested as decreased membrane receptor density (13, 20), G protein content, and adenylyl cyclase activity (19, 21, 20, 22), 2) decreased membrane fluidity, which hinders the receptor-ligand and G protein coupling process (19, 20), and 3) increased inducible NOS levels with stimulation of the negative inotropic second messenger cGMP (16). The present results now indicate that yet another pathway acting through the cardiac-inhibitory cGMP system is overexpressed. On the other hand, it is known that cirrhotic cardiomyopathy tends to be relatively mild and is only unmasked by physiological, surgical, or pharmacological stress. Indeed, BDL rats (and humans with cirrhosis) show increased cardiac output at rest. One might then reasonably wonder why overt severe ventricular failure is rare in cirrhosis, if there are so many attenuated stimulatory influences and increased inhibitory factors. We believe that the explanation is twofold. First, many of the cardiac-inhibitory factors such as increased cGMP and decreased cAMP also concomitantly induce peripheral vasodilatation. Such vasodilatation unloads the ventricle, in effect “autotreating” the mild ventricular failure. Second, we have also found a partially compensatory reduction in some other inhibitory influences such as the cardiac muscarinic cholineric system (12) and cardiomyocyte membrane G protein (G inhibitory protein that attenuates contractility) content (21). One might also wonder why ZnPP treatment in the present study restored cGMP levels and isolated papillary muscle contractility to sham-operated control values if there are other cardiodepressant systems at play. We believe that the explanation lies in the in vitro methods of our study, which allow the full expression of a single manipulation to show effects. It is evident that such an in vitro system would not directly correspond to the actual in vivo regulation of contraction with its intact neural, humoral, and other local regulatory factors present.

The present study as well as most of the others discussed above were done in the deeply jaundiced BDL cirrhotic rat model. This may complicate interpretation of the data because deep jaundice per se may have some cardiodepressant effects (11). However, we believe that the jaundice did not play a significant role in the current results for several reasons. First, studies by Bomzon and colleagues (11) showed that short-term BDL rats (3 days after ligation), which have deep jaundice but no cirrhosis, develop an entirely different pattern of cardiovascular anomaly, with intact cardiac β-adrenoceptor density and function. They also showed that bile salts at the levels seen in BDL rats do not affect cardiac β-adrenergic receptor characteristics or function (6). Finally, and perhaps most significantly, our previous work (22) demonstrated that ventricular β-adrenergic responsiveness patterns are similar in three disparate cirrhotic rat models irrespective of the level of jaundice and that the mild cardiodepressant effects of jaundice are caused by a direct inhibitory effect of jaundice on the adenylyl cyclase enzyme itself. Therefore, the evidence is overwhelming that cirrhosis per se, rather than jaundice, is the prime determinant of impaired cardiac function in cirrhotic cardiomyopathy.

In conclusion, the current results indicate that activation of the HO-CO pathway occurs in cirrhotic rat hearts, with expression of HO-1, and that abrogation of CO effects decreases the second messenger cGMP production and improves cardiac contractility. CO appears to mediate its cardiodepressant effects via stimulation of cGMP. These results suggest that increased CO production due to HO activation may play an important role in the pathogenesis of cirrhotic cardiomyopathy.

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