Original article

Reduced heme oxygenase-1 expression in steatotic livers infected with hepatitis C virus

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Hepatic nonalcoholic fatty liver disease (NAFLD) is known to exacerbate liver injury due to chronic hepatitis C infection. Heme oxygenase-1 (HO-1) is an important protective antioxidative defense enzyme that is known to be induced in response to NAFLD and other liver injuries. The aim of this study was to evaluate HO-1 expression in HCV infected human livers with concomitant NAFLD.

Methods: We compared levels of HO-1 in NAFLD liver biopsies from patients with or without chronic HCV infection using immunohistochemistry, immunoblots and real time RT-PCR. We also evaluated frozen sections of liver with dihydroethidium (DHE) or dichlorofluorescein (DCF) fluorescence staining to evaluate O2− and peroxide production respectively.

Results: HO-1 expression was only increased in NAFLD livers without HCV infection, while HCV infected livers showed reduced HO-1 levels, regardless whether NAFLD was present. In uninfected livers with NAFLD, HO-1 expression was primarily localized in hepatocytes containing fat and areas of injury around the central vein. However, both NAFLD with and without concomitant HCV infection showed high levels of O2− or peroxide production compared to normal human liver control samples.

Conclusions: These findings support the hypothesis that NAFLD is an important process for hepatocyte oxidative stress and injury in liver diseases. They also suggest that HCV can repress HO-1 induction in vivo even when other inducers of HO-1 are present.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a serious medical problem worldwide. The spectrum of NAFLD includes uncomplicated NAFLD as well as steatohepatitis leading to cirrhosis. It is currently estimated that 20% of the US population has NAFLD with the more serious Non Alcoholic Steatohepatitis (NASH) present in up to 20% of all obese people [1,2]. NASH was described by Ludwig in 1980 as liver disease that histologically mimics alcoholic hepatitis but occurs in patients without significant alcohol consumption [3,4]. Risk factors for NASH include elevated BMI, hyperlipidemia, and type II diabetes with metabolic syndrome [5]. NASH is characterized historically by aggressive fatty infiltration of hepatocytes, hepatocellular injury with inflammation, and fibrosis [3].

Chronic Hepatitis C virus infection (HCV) is also a major cause of cirrhosis and end stage liver disease worldwide [6,7]. In general, about 20% of patients infected with HCV progress to cirrhosis, however, accessory patient factors such as obesity, type II diabetes, and NAFLD are known to accelerate HCV disease progression [8,9]. Consequently, patients with NAFLD and concomitant HCV infection are more likely to have progressive fibrotic liver disease than patients with either disease alone. The relationship between HCV and hepatic NAFLD, potentially synergistic, appears complex and is poorly understood at the molecular level. NAFLD in chronic HCV infection can arise primarily by the virus, especially genotype 3 [10], or it can arise independently from other patient risk factors such as hyperlipidemia and type II diabetes [11,12].

In both HCV infection and NAFLD, fat accumulation in the hepatocyte is a major instigator of intracellular damage [13,14]. Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), HCV, and lipid peroxidation play an important role in liver pathogenesis from either disease entity [15–17]. ROS or RNS may be responsible for the progression from simple fatty liver to NASH [18–20].

Abbreviations: HCV, hepatitis C virus; HO, heme oxygenase; RT-PCR, Reverse Transcriptase Polymerase Chain Reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WB, Western blot; CV, coefficient of variation; ANOVA, analysis of variance; DHE, dihydroethidium; DCF, dichlorofluorescein (2,7 dichlorofluorescein diacetate); ROS, Reactive Oxygen Species; RNS, Reactive Nitrogen Species; NAFLD, Nonalcoholic fatty liver disease.

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Anti-oxidative enzymes are induced in response to oxidative stress and are considered a primary cellular defense mechanism against free radical and oxidative injury [21,22]. Heme oxygenase-1 (HO-1) is a well characterized, anti-oxidative enzyme that is induced in response to cellular injury from numerous agents such as cytokines, heavy metals, reactive oxygen species (ROS), and protumour IX [23–25]. In the liver, HO-1 is typically induced in response to injury from diseases such as autoimmune hepatitis [26,27], chronic hepatitis B infection [27,28], and NASH [29]. Furthermore, recent studies have shown that HO-1 induction or overexpression can attenuate intracellular injury from HCV infection through antiviral and anti-oxidative activities of the enzyme’s reaction products, iron and biliverdin [30–32]. However, HO-1 is down-regulated in the livers from patients with HCV infection, suggesting that the usual antioxidative HO-1 defense mechanism of the hepatocyte is specifically impaired by the virus causing the hepatocyte to be more prone to oxidative injury [27,33,34].

In the present report, we studied the influence of hepatic NAFLD on HO-1 expression and cellular damage in livers from HCV infected patients. While NAFLD without HCV infection promoted increased HO-1 expression, HO-1 expression remained repressed in patients with both NAFLD and chronic HCV infection. These findings suggest a plausible mechanism for the increased cellular injury when both disease entities are present.

2. Materials and methods
2.1. Biopsy samples
Liver biopsy samples from patients with non-alcoholic fatty liver disease (NAFLD or steatosis), HCV, HCV with NAFLD, and normal liver controls were collected and processed as described [27]. All experimental samples were obtained from subjects that provided written informed consent. The study was approved by the University of Iowa Institutional Review board.

2.2. Western blotting
HO-1, catalase and Mn superoxide dismutase (MnSOD) were employed as described [27]. Briefly, at least 3 liver biopsy samples were pooled and homogenized in the presence of Tris buffer (50 mM, pH 7.4) containing 1% Nonidet P-40 (Amresco, Solon, OH) and 0.1 mmol phenylmethylsulfonyl fluoride (PMSF)/L. Tissue lysates were collected by centrifugation. Samples (20–30 μg protein) were mixed 1:1 with sample buffer (1.25 M Tris, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.05% bromophenol blue). Proteins were separated by 12% SDS-PAGE and blotted to a nitrocellulose membrane. The membrane was blocked and incubated with the primary antibody for 1–2 h. Secondary antibody was horseradish peroxidase-conjugated anti-IgG (Amersham Pharmacia Biotech). The immunoreactive protein was detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

2.3. Immunohistochemistry
HO-1 localization has been described [27]. Briefly, paraffin embedded liver tissues were cut into 6-μm thick sections. Representative sections were deparaffinized, rehydrated, and treated with 3 M urea in a pressure cooker for 10 min for antigen retrieval. Sections were incubated with antibody to HO-1 (1:1000 dilution for 30 min), washed, and then incubated with labeled secondary antibody. Immunoreactivity was visualized with the Vectorstain ABC kit using 3-amino-9-ethylcarbazole or 3,3-diaminobenzidine tetrahydrochloride substrate as the final chromogen (Vector Laboratories, USA). Sections were counterstained with Vector hematoxylin. Negative controls included sections stained after omission of the first antibody or after incubation with mouse immunoglobulin as the primary antibody.

2.3.1. Assessment of intracellular: O$_2^-$ and H$_2$O$_2$ levels using dihydroethidium (DHE) and 2,7-dichlorofluorescein diacetate (H$_2$DCF-DA) staining

*In situ* staining of O$_2^-$ and H$_2$O$_2$ was performed using frozen human liver biopsy sections as described [35]. Fresh human liver biopsy samples (2–4 mm long) were obtained as excess material from 3 cm routine patient liver biopsies. Tissue was immediately frozen in Tissue Tec and stored at –70 °C until use. Frozen liver tissue sections (20–30 μm) were prepared for *in situ* imaging of O$_2^-$ and H$_2$O$_2$ with the fluorescent dyes DHE, and H$_2$DCF-DA (Molecular Probes, Inc, USA). The oxidative fluorescent dye DHE is freely permeable to cells and in the presence of O$_2^-$ is oxidized to ethidium bromide (EtBr), which is trapped by intercalating with DNA. EtBr is excited at 488 nm with an emission spectrum of 610 nm [36]. Intracellular production of H$_2$O$_2$ was measured by using H$_2$DCF-DA. This nonpolar compound is converted to the membrane-impermeant polar derivative H$_2$DCF by esterases when it is taken up by the cell. H$_2$DCF is nonfluorescent but is rapidly oxidized to the highly fluorescent DCF by intracellular H$_2$O$_2$ and other peroxides [37]. Unfixed frozen liver sections were cut into 30-μm-thick sections and placed on a glass slide. H$_2$DCF-DA was added to sections at a final concentration of 5 μM and DHE at 10−5 M. Slides were incubated for 30 min in a humidified chamber at 37 °C. Images were obtained with a Zeiss 510 laser scanning confocal microscope.

2.3.2. Real-time RT-PCR
Purified RNA from liver tissue samples was measured and used for Real-Time RT-PCR. Primers used were for real-time RT-PCR quantitation of HO-1 mRNA were sense 5′-AGG-GTG-ATA-AGAG-GGC-AAG-AC-3′ (nucleotides 697–720), antisense 5′-TG-CAG-TAAC-TCA-AGG-AGC-3′ (nucleotides 741–761), Real-time RT-PCR was performed as previously described using TaqMan 18S Ribosomal RNA control [38]. HO-1 mRNA was quantitated using the TaqMan system (Perkin Elmer Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Relative quantitative analysis was determined by the comparative cycle threshold (Ct) method, which used separate reaction tubes for target (HO-1) and reference (18S rRNA) sequences. Validation experiments showed similar amplification efficiencies for the target gene and the 18S amplicons. The comparative Ct method was used to calculate the amount of target mRNA, normalized to the amount of 18S RNA, and relative to an internal calibrator (control human liver), expressed as 2−ΔΔCt [33].

2.3.3. Data analysis
Data from separate experiments are expressed as mean ± standard error of the mean (SEM). The statistical significance of observed differences between means was determined using Student’s *t*-test (for comparing the means of two samples) or ANOVA (for comparing means of 3 or more samples) and was defined as p<0.05.

3. Results
3.1. HO-1 expression in liver biopsies from NAFLD, HCV patients and normal controls

The protein expression of HO-1 in liver biopsies from patients with NAFLD, HCV, and NAFLD/HCV patients was determined and compared to normal control liver tissues using immunohistochemistry, immunoblot analysis, and RT-PCR. We first evaluated paraffin-embedded human liver sections immunohistochemically (Fig. 1). HO-1 immunostaining was noticeably higher in NAFLD liver biopsies as compared to control biopsies (Fig. 1 A and B). In contrast, NAFLD with HCV infection did not show increased HO-1 expression [Fig. 1].
A and E). As noted previously [27], HO-1 expression was decreased in HCV infected liver compared to control liver (Fig. 1C and D).

The expression of HO-1 was further studied at the protein level using western blots of solubilized liver biopsies. As shown in Fig. 2, the level of HO-1 protein was higher in liver tissues from patients with NAFLD (39%) compared to normal controls. In contrast, HO-1 level in HCV infected tissues was 16% lower than that of the normal control.

To examine whether the change is the protein level only or extends to the gene level, quantitative real time RT-PCR was performed on isolated RNA from various human liver samples. These experiments revealed that HO-1 mRNA from biopsies with increased NAFLD (grade 3) was higher than normal control liver sections (Fig. 3). In contrast, HCV infected liver samples with NAFLD showed significantly less HO-1 mRNA and interestingly, there was a progressive decrease as a function of NAFLD grade (Fig. 4).

3.2. Increased ROS production in NAFLD and HCV

NAFLD and HCV are associated with increased ROS production and oxidative stress. To explore ROS production in liver biopsies with NAFLD and HCV, fresh liver biopsies were stained for in situ production of ROS using the $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ sensitive probes; DHE and/or DCF, respectively.

As shown in Fig. 5, the production of $\text{O}_2^-$ was increased in biopsies with NAFLD as indicated by the increase in the green fluorescence (DCFH) staining compared to control (Fig. 5A and F). When liver biopsy sections were co-stained with HE and DCFH, increased fluorescence of both colors (red and green) was seen compared to controls (Fig. 5B and F). Similarly, liver biopsies with HCV and NAFLD showed higher green and red fluorescence when stained with DCF and HE indicating increased $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ production compared to controls (Fig. 5C and F).

3.3. Antioxidant enzymes expression in NASH, HCV and control liver tissues

Increased HE and DCFH fluorescence led us to study the expression of major antioxidant enzymes such as MnSOD and Catalase. MnSOD catalyzes the dismutation of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and $\text{O}_2$. Catalase is a heme containing redox enzyme. It is found in high concentrations in peroxisome. It scavenges $\text{H}_2\text{O}_2$ to produce water and $\text{O}_2$. 

Fig. 1. HO-1 expression in NAFLD and HCV liver biopsies by immunohistochemistry. HO-1 immunohistochemical staining was performed as described in materials and methods. A) Liver biopsy with NAFLD showing increased HO-1 (brown color). B) Negative control was performed by omitting the primary antibody. C) Liver biopsy sample from patient with HCV without NAFLD. D) Liver biopsy sample from normal control liver. E) Liver biopsy sample from patient with HCV and stage 2 NAFLD. Solid arrows show HO-1 stain in hepatocytes and open arrows show stained HO-1 in Kupffer cells.
Our results show that MnSOD expression level in NAFLD was unchanged compared to normal controls. A similar result was noted for liver biopsies from patients with NAFLD and HCV (Fig. 6). The expression of catalase, on the other hand, was increased (58%) in NAFLD livers compared to controls (Fig. 6). In liver biopsies from patients with NAFLD and HCV, catalase was increased (26%) compared to controls (Fig. 6).

4. Discussion

Non-alcoholic hepatic steatosis is common disorder characterized by fatty infiltration of hepatocytes. Hepatic NAFLD can range from a relatively benign condition to progressive severe end stage liver disease with the need for liver transplantation [3]. It is believed that oxidative stress has a significant role in the pathogenesis of hepatic NAFLD, causing damage by protein and lipid peroxidation leading to altered gene expression, inflammation and apoptosis [19,39,40]. Heme oxygenase-1, an important antioxidative defense enzyme, has been reported to be upregulated in the liver as a result of liver injury due to NAFLD and other liver diseases [29,41,42]. In contrast, intrahepatic levels of HO-1 were reported to be reduced in HCV infected liver [27] and this has been suggested to be in part responsible for HCV induced liver injury. The overall aim of the present study was to determine whether concomitant HCV infection influenced increased in vivo expression of HO-1 due to NAFLD.

Fatty liver and steatohepatitis have been shown to be accompanied by increased HO-1 expression in steatohepatocytes [41]. Our results shows that the effect of NAFLD on HO-1 is on the protein level and extended to the gene level as shown by immunohistochemistry, Western blots and RT-PCR (Figs. 1, 2, and 3). Others have reported similar results [29,41,43]. The HO-1 localization and distribution were confirmed by immunohistochemistry (Fig. 1).

The cytoprotective effect of HO-1 has been described, and its role in preventing liver injury from inflammatory and oxidative damage has been implied [30,31]. HO-1 induction in response to various insults would prevent tissues from production of oxidative damage and pro-inflammatory cytokines, thereby inhibiting liver inflammation [41]. Others proposed that HO-1 downregulation in certain diseases could protect against the potential toxic consequences of increased cellular free iron [44].

A connection between HO-1 and HCV was established by the finding that HCV apparently interferes with HO-1 induction, resulting in increasing ROS production, oxidative stress and cytotoxicity [33]. Furthermore, different viral proteins have the capacity to regulate cellular defense antioxidative activities differentially. Both HCV core, and non-structural proteins such as NS5a, have been shown to modulate oxidative stress [26,27,33]. In addition, it has been shown that HO-1 enzymatic products: biliverdin [45] and iron mediated anti-inflammatory and oxidative damage [43]. Studies have shown that HO-1 downregulates HO-1 expression due to NAFLD. A summary of these studies is shown in Fig. 4.

NAFLD is frequently seen in the setting of chronic HCV infection and is an independent risk factor for severe disease progression when present [8]. However, previous to the present study, the influence of HCV on HO-1 expression when NAFLD is present has not been reported. We have shown that the presence of HCV interferes with the induction of HO-1 in NAFLD liver biopsies. The effect of HCV was seen immunohistochemically and supported at the protein and mRNA levels. We have shown before that HCV downregulates HO-1 in HCV liver biopsies as compared to normal controls [27]. It has also been shown by our group and others that HO-1 has antiviral effects on HBV, HCV and HIV [28,30,46]. It is interesting to note that the increase in NAFLD levels in the HCV infected patients increases the down regulation of HO-1 (Fig. 4). It is possible that NAFLD plays a major role in reducing the HO-1 expression in HCV infected patients, while, normally, it up-regulates the HO-1 enzyme. Similar observation was reported by Stoll P. et. al. [47] who suggested a possible...
role of steatosis in preventing HO-1 induction by isoflurane in the rat liver. They demonstrated that isoflurane is a potent inducer of HO-1 gene expression in non-steatotic livers but failed to upregulate HO-1 in steatotic organs. However, further investigation is still needed to confirm the correlation between NAFLD and HO-1 down-regulation in HCV infected patients [47].

HCV associated oxidative stress and increased production of ROS are believed to cause liver damage seen in chronic HCV infection [32,48]. Multiple studies have shown increased lipid peroxidation and reduced glutathione concentrations in patients with HCV-infection [40,49]. ROS likely arises from inflammatory cells as well as hepatocytes as a result of different HCV protein expressions [26,33,34,50].

Our in vivo results showed increased ROS production during HCV infection and NAFLD compared to control liver. The increased fluorescence after HE or DCFH indicates that ROS activity associated with these disease entities contains both $O_2^-$ and $H_2O_2$. Furthermore, while the expression of major antioxidant defense enzymes such as manganese superoxide dismutase (MnSOD) and Catalase were not overtly changed, HO-1 expression was increased only in NAFLD without HCV infection. These results support our past studies which demonstrated that HO-1 expression was not only down-regulated in HCV infected livers, but that HO-1 induction in response to stimuli is impaired in vitro [30,33]. Collectively, our data as well as those of others indicate that HCV has a powerful ability to regulate HO-1 expression [30,33,45,48]. An impaired response to HCV oxidative stress may be a significant factor in HCV induced liver injury. At the cellular level, HO-1 overexpression or induction with hemin afforded protection to cellular damage from the virus [30,45]. Oxidative species that are responsible for oxidative stress during HCV infection
likely arise from multiple sites such as different viral proteins and viral activities such as replication and assembly [26,48]. The HCV nucleocapsid core protein depletes reduced cellular thiol stores which are not compensated by increases in catalase or MnSOD [26]. In contrast, non-structural HCV proteins, although a source of oxidants, upregulate MnSOD and catalase and maintain reduced glutathione levels adequately [26,33,50].

Conclusion

In summary, our findings support the work of others that have shown that HO-1 expression is usually upregulated in response to various human liver diseases including NAFLD. However, when HCV is present with NAFLD; HO-1 expression is suppressed. Our study provides clear evidence in humans that the HO-1 enzyme increased significantly in liver biopsies from NAFLD and the presence of HCV suppresses this induction. This could be through direct or indirect interaction of HCV different proteins in the HO-1 induction pathway (Fig. 7).

Learning points

• HO-1 is an indicator of oxidative stress and protective enzyme in infection.
• NAFLD increase HO-1 expression in liver cells.
• The presence of HCV prevents HO-1 upregulation and increase cellular injury.

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Conflict of interest

None of the authors who participated in this study has commercial or other associations that might pose a conflict of interest.

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