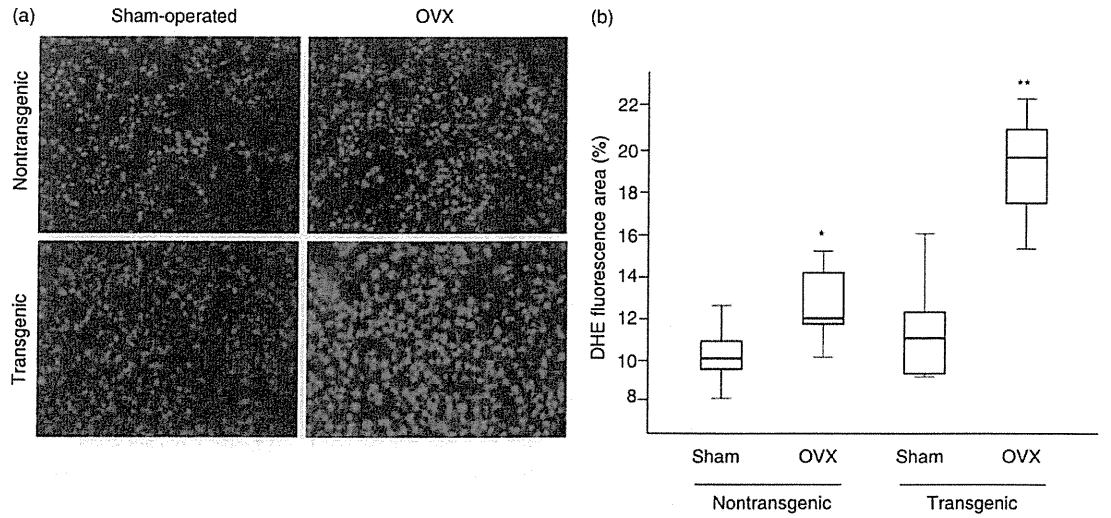

Figure 2 Reactive oxygen species (ROS) production in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) Frozen liver sections from mice in each group were stained with dihydroethidium (DHE). (b) Fluorescence intensity was quantified by NIH image analysis software for three randomly selected areas of digital images for five mice in each group. The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the line within each box. *: $P < 0.05$ versus sham-operated non-transgenic mice. **: $P < 0.05$ versus sham-operated nontransgenic mice, OVX non-transgenic mice and sham-operated transgenic mice.

oxide) production in both transgenic mice and non-transgenic mice, but the level of ROS production was greater in the OVX transgenic mice than in the OVX non-transgenic mice (Fig. 2). We next measured inflammatory cytokine levels in the liver. Ovariectomy significantly increased hepatic expression of IL-6 mRNA to the same degree in both transgenic mice and non-transgenic mice (Fig. 3). This ovariectomy-induced increase in hepatic IL-6 mRNA was consistent with the results of a previous report that OVX mice produced more hepatic

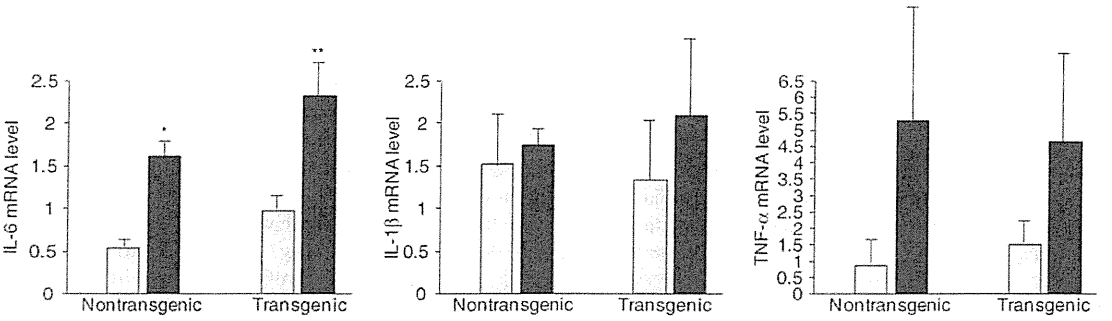

Figure 3 Expression levels of inflammatory cytokines in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. The mRNA levels of interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α were measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. *$P < 0.05$ vs sham-operated non-transgenic mice. **$P < 0.05$ vs sham-operated transgenic mice. □, Sham; ■, OVX.

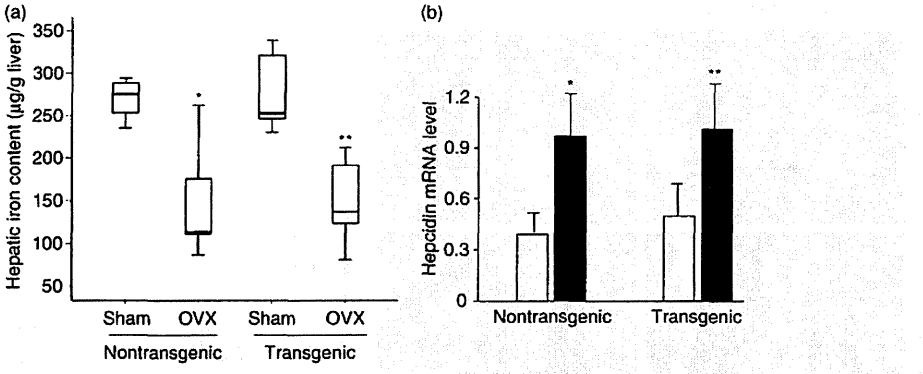

Figure 4 Hepatic iron content and hepcidin mRNA level in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) Hepatic iron content in mice in each group ($n = 5$). The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the line within each box. $^{*}P < 0.05$ vs sham-operated non-transgenic mice. $^{**}P < 0.05$ vs sham-operated transgenic mice. (b) The mRNA level of hepcidin was measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. $^{*}P < 0.05$ vs sham-operated non-transgenic mice. $^{**}P < 0.05$ vs sham-operated transgenic mice. □, Sham; ■, OVX.

IL-6 than non-OVX mice after chemically induced liver injury.[5] There also was a trend for increase in TNF-α and IL-1β mRNA expression after ovariectomy in both the transgenic mice and non-transgenic mice, but their increases did not reach statistical significance, probably because of the large deviation (Fig. 3). These results suggested that inflammatory cytokines were unlikely to be associated with greater ROS production in OVX transgenic mice than in OVX non-transgenic mice.

Hepatic iron content and hepcidin expression level in the liver

We previously reported that male FL-N/35 transgenic mice developed hepatic iron accumulation through the reduced transcription of hepcidin,[18] a negative regulator in iron homeostasis.[21,22] Excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals.[23] Therefore, we measured hepatic iron content to assess whether greater ROS production resulted from increased hepatic iron accumulation in OVX transgenic mice. Unexpectedly, ovariectomy significantly decreased hepatic iron content to the same degree in both transgenic mice and non-transgenic mice (Fig. 4a). These results are potentially explained by significantly increased transcription of hepcidin after ovariectomy (Fig. 4b). Ovariectomy-induced increase in hepatic IL-6 mRNA may in turn account for increased hepcidin transcription, because IL-6 acts to stimulate hepcidin expression through the STAT3 pathway.[24] These results suggested that hepatic iron content was not related to greater ROS production in OVX transgenic mice than in OVX non-transgenic mice.

Attenuated antioxidant potential against ovariectomy-induced ROS production in FL-N/35 transgenic mice

The increase in inflammatory cytokine production and the hepatic iron content after ovariectomy were comparable in transgenic and non-transgenic mice. Nevertheless, the serum ALT level, hepatic steatosis and ROS production were greater in OVX transgenic mice than in OVX non-transgenic mice. Therefore we measured dROM and BAP in serum to compare antioxidant potentials in OVX transgenic and OVX non-transgenic mice. We confirmed the significant negative correlation between the ratio of BAP to dROM and hepatic content of superoxide (Fig. 5). As expected, the values for dROM were higher in OVX mice than in sham-operated mice, regardless of whether they were transgenic or non-transgenic. However, a significant increase in the BAP value was found in OVX non-transgenic mice but not in OVX transgenic mice, which resulted in a lower ratio of BAP to dROM in the OVX transgenic mice than in the OVX non-transgenic mice (Table 2).

The first line of defense against ROS is the detoxifying enzymes that scavenge ROS. These include SOD and

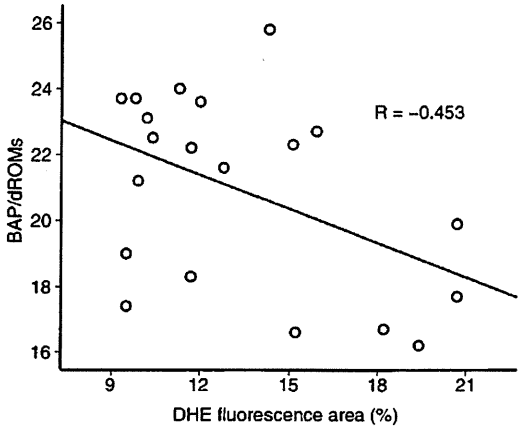

Figure 5 Negative correlation between the ratio of biological antioxidant potential (BAP) to derivatives of reactive oxygen metabolites (dROM) and hepatic content of superoxide. $R = -0.453$, $P < 0.05$. Hepatic content of superoxide was determined based on the area of dihydroethidium (DHE) fluorescence.

GPx1. Therefore we next investigated the expression levels of SOD2 and GPx1. The hepatic expression levels of SOD2 mRNA and GPx1 mRNA were significantly greater in OVX non-transgenic mice than in sham-operated non-transgenic mice, but were comparable in OVX transgenic mice and sham-operated transgenic mice (Fig. 6a). Western blot analysis of the hepatic mitochondria fractions also showed significant increases of SOD2 and GPx1 expression in OVX non-transgenic mice but not in OVX transgenic mice (Fig. 6b). These results suggested that antioxidant defense mechanisms may be induced against ovariectomy-related ROS production in non-transgenic mice but not in transgenic mice.

SIRT3 and PGC-1α expression in OVX FL-N/35 transgenic mice

Proliferator-activated receptor-γ co-activator-1α is a master regulator of mitochondrial biogenesis and respiration[25] and required for the induction of many ROS-detoxifying enzymes, including SOD2 and GPx1 upon oxidative stress.[26] SIRT3 is a member of a class III histone deacetylase and is reported to mediate PGC-1α-dependent induction of ROS-detoxifying enzymes.[27] In accordance with the changes in SOD2 and GPx1 levels after ovariectomy, the hepatic expression of SIRT3 mRNA was significantly greater in OVX non-transgenic mice than in sham-operated non-transgenic mice, but comparable in OVX transgenic mice and sham-operated transgenic mice (Fig. 7a). Western blot analysis of hepatic mitochondria showed a significant increase of SIRT3 expression in OVX non-transgenic mice but not in OVX transgenic mice (Fig. 7a).

Proliferator-activated receptor-γ co-activator-1α interacts with various nuclear receptors in addition to peroxisome proliferator-activated receptor-γ and is docked to the promoter of its target genes by all these nuclear receptors. Therefore, we investigated PGC-1α expression levels not only in liver homogenates but also in the nuclear fraction of mouse liver. The expression levels of PGC-1α in liver homogenates were comparable in sham-operated and OVX non-transgenic mice and in sham-operated and OVX transgenic mice. However, the expression levels of PGC-1α in the nuclear fraction of the liver significantly increased after ovariectomy in both non-transgenic and transgenic mice, and OVX transgenic mice had a lower PGC-1α expression level than OVX non-transgenic mice (Fig. 7b). These results suggested that the antioxidant potential against ovariectomy-induced ROS production may be reduced in OVX transgenic mice through lesser activation of PGC-1α than in OVX non-transgenic mice.

Table 2 Derivatives of reactive oxygen metabolites (dROM), biological antioxidant potential (BAP) and ratio of BAP to dROM

	Non-transgenic		Transgenic	
	Sham-operated	OVX	Sham-operated	OVX
dROM (U.CARR)	145.2 ± 15.1	158.7 ± 15.9*	170.8 ± 10.4	199.3 ± 21.1**
BAP (μmol/L)	3217 ± 123	3644 ± 177*	3362 ± 178	3542 ± 140
Ratio of BAP to dROM	22.3 ± 2.3	23.1 ± 2.0	20.8 ± 1.8	17.8 ± 1.9***

Data are mean ± standard deviation.
*$P < 0.05$ compared with sham-operated non-transgenic mice. **$P < 0.05$ compared with sham-operated transgenic mice. ***$P < 0.05$ compared with ovariectomized (OVX) non-transgenic mice.

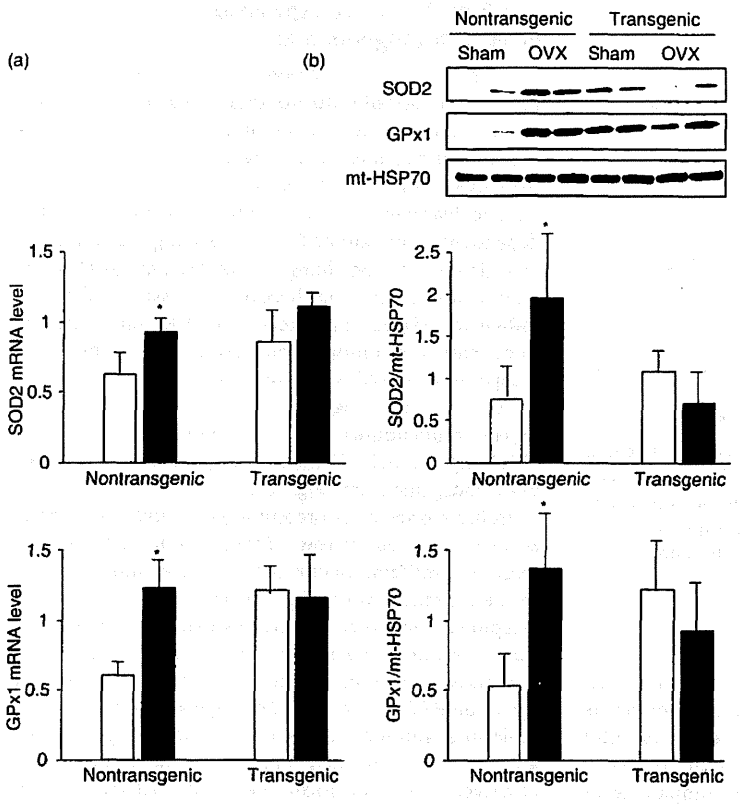

Figure 6 Expression levels of superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPx1) in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) The mRNA levels of SOD2 and GPx1 were measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. (b) Immunoblots for SOD2 and GPx1 were performed using mitochondrial fractions of liver lysates from five mice in each group. $^*P < 0.05$ vs sham-operated non-transgenic mice. □, Sham; ■, OVX.

Suppressed AMPK activation in OVX FL-N/35 transgenic mice

Proliferator-activated receptor-γ co-activator-1α activity is modulated through both transcriptional regulation and regulation of its activity by post-translational modifications.[28] AMPK is one of the signaling pathways regulating PGC-1α and acts both through modulation of PGC-1α transcription and by phosphorylation of the PGC-1α protein.[28] HCV has been shown to reduce the kinase activity of AMPK through Ser485/491 phosphorylation of AMPK.[29] Therefore, we examined the expression levels of AMPK to investigate the mechanisms underlying the lower PGC-1α expression in the nuclear fraction of the OVX transgenic liver. The expression levels of AMPKα, which is one of the three subunits (α, β and γ) of AMPK, were comparable in sham-operated and OVX mice and in non-transgenic and transgenic mice. However, the expression level of phosphorylated AMPKα was significantly greater in OVX non-transgenic mice than in mice in the three other groups, though it was similar in sham-operated transgenic mice and OVX transgenic mice (Fig. 7c). In addition, its levels were significantly greater in non-transgenic mice than in transgenic mice (Fig. 7c). These results suggested that AMPK was activated in OVX non-transgenic mice, but not in OVX transgenic mice, because AMPK is active only after phosphorylation of the α-subunit at a threonine residue within the kinase domain (T172) by upstream kinases.[30] Taken together, the results in the present study suggested that OVX FL-N/35 transgenic mice developed marked hepatic steatosis concomitant with increased ROS production via attenuation of antioxidant potential through inactivation of the AMPK/PGC-1α signaling pathway.

DISCUSSION

THE OVX MICE in the present study were assumed to be a standard model for evaluating the biological effect of ovariectomy because the effects of ovariectomy

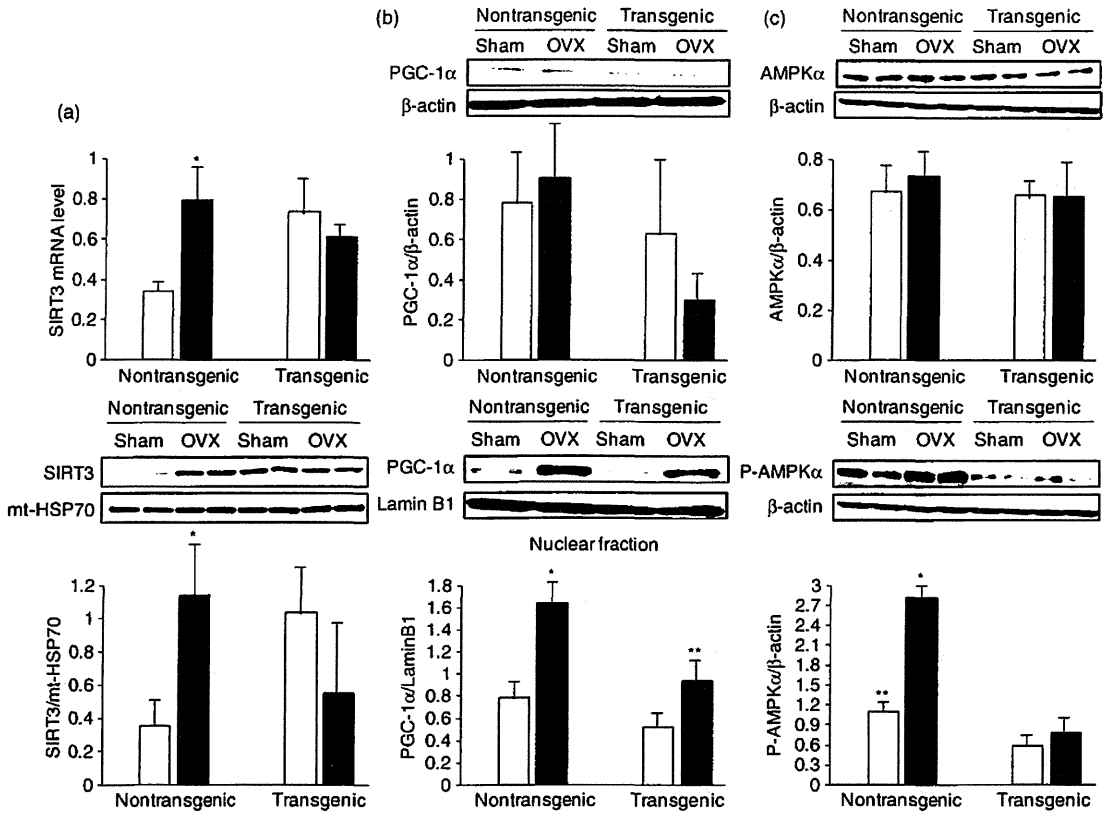

Figure 7 Expression levels of sirtuin 3 (SIRT3), peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), adenosine monophosphate-activated protein kinase α (AMPKα), and phosphorylated AMPKα (P-AMPKα) in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) The mRNA levels of SIRT3 were measured by real-time reverse transcription polymerase chain reaction for five mice in each group. The relative quantities of target mRNA in the liver were normalized with GAPDH mRNA. Immunoblots for SIRT3 were performed using the mitochondrial fractions of liver lysates from five mice in each group. (b) Immunoblots for PGC-1α were performed using liver lysates and their nuclear fractions from five mice in each group. $^{*}P < 0.05$ vs mice in the other three groups. $^{**}P < 0.05$ vs sham-operated transgenic mice. (c) Immunoblots for AMPKα and P-AMPKα were performed using liver lysates from five mice in each group. $^{*}P < 0.05$ vs mice in the other three groups. $^{**}P < 0.05$ vs sham-operated transgenic mice. □, Sham; ■, OVX.

on dietary intake, bodyweight, uterine weight, liver weight and serum leptin levels were similar to the results from previous studies.[31–34] Ovariectomy increased ROS (superoxide) production in both transgenic liver and in non-transgenic liver, which was consistent with the ovariectomy-induced increase in NADPH oxidase activity[12] and the protective effect of estrogen against mitochondrial oxidative damage[13] found in previous studies. Of note was the much greater degree of ROS production after ovariectomy in transgenic mice than in non-transgenic mice. These results suggested that HCV protein expression has the potential to increase the sensitivity to oxidative stress in the liver. At least two possibilities may account for the increased sensitivity to oxidative stress in FL-N/35 transgenic mice. One possibility is an additive effect of HCV-induced ROS production on ovariectomy-induced oxidative stress. The HCV core protein has been shown to inhibit mitochondrial electron transport[35] and to induce ROS production.[36] In fact, basal ROS production tended to be higher in

transgenic mice than in non-transgenic mice, but was not significantly different. These results suggested that additive HCV-induced ROS production was unlikely to be the cause of the significantly increased ROS production after ovariectomy in the transgenic mice. The other possibility is HCV-associated attenuation of antioxidant potential against ovariectomy-induced oxidative stress. In this respect, OVX transgenic mice had a lower ratio of BAP to dROM than OVX non-transgenic mice and the expression of SOD2 and GPx1 in the liver was not increased. These results suggest that HCV protein attenuated antioxidant potential against ovariectomy-induced oxidative stress.

Proliferator-activated receptor-γ co-activator-1α is required for the induction of many ROS-detoxifying enzymes upon oxidative stress.[26] SIRT3 has been shown to function as a downstream target gene of PGC-1α and mediate the PGC-1α-dependent induction of ROS-detoxifying enzymes.[27] Additionally, AMPK, which is a crucial cellular energy sensor, regulates PGC-1α activity through both modulation of PGC-1α transcription and phosphorylation of the PGC-1α protein.[28,17] Thus, AMPK/PGC-1α signaling is one of the important pathways that protect cells from oxidative stress through the induction of several key ROS-detoxifying enzymes. Recent evidence indicating that HCV replication inhibits AMPK activity[29] prompted us to investigate whether the antioxidant potential against ovariectomy-induced oxidative stress in FL-N/35 transgenic mice was attenuated through inhibition of this signaling pathway. As expected, upon ovariectomy, AMPK was activated in non-transgenic mice, but not in transgenic mice. This, in turn, led to the lower expression of PGC-1α in the nuclear fraction of the liver in OVX transgenic mice than in OVX non-transgenic mice, resulting in the absence of significant induction of SIRT3 in the mitochondrial fraction of the liver in the OVX transgenic mice. Thus, ROS production in the liver in OVX transgenic mice was increased by attenuation of the antioxidant potential through inhibition of AMPK/PGC-1α signaling. However, it remains unknown why the expression of PGC-1α in the nuclear fraction was significantly increased in OVX transgenic mice regardless of the lack of activation of AMPK. Various kinases other than AMPK and post-translational modifications other than phosphorylation have been shown to regulate PGC-1α expression.[28] Therefore further investigations are required to clarify this issue.

Of particular concern is the relevance of the present results to HCC development in patients with HCV-associated chronic liver diseases. A recent study from Japan demonstrated a higher proportion of females, especially among elderly patients with HCV-related HCC, suggesting that the sex disparity in HCC development becomes less distinct as the patient's age at HCC diagnosis increases.[6] In general, ROS production creates a pro-carcinogenic environment under which chromosomal damage is likely to occur. The present findings that OVX transgenic mice have increased hepatic ROS production compared with that in OVX non-transgenic mice may indicate one of the mechanisms by which women with HCV infection are at high risk for HCC development when some period has passed after menopause, even though we need to clinically ascertain the increased hepatic oxidative stress in HCV-infected menopausal women with HCC.

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Sofosbuvir plus ribavirin in Japanese patients with chronic genotype 2 HCV infection: an open-label, phase 3 trial

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SUMMARY. Genotype 2 hepatitis C virus (HCV) accounts for up to 30% of chronic HCV infections in Japan. The standard of care for patients with genotype 2 HCV – peginterferon and ribavirin for 24 weeks – is poorly tolerated, especially among older patients and those with advanced liver disease. We conducted a phase 3, open-label study to assess the efficacy and safety of an all-oral combination of the NS5B polymerase inhibitor sofosbuvir and ribavirin in patients with chronic genotype 2 HCV infection in Japan. We enrolled 90 treatment-naïve and 63 previously treated patients at 20 sites in Japan. All patients received sofosbuvir 400 mg plus ribavirin (weight-based dosing) for 12 weeks. The primary endpoint was sustained virologic response at 12 weeks after therapy (SVR12). Of the 153 patients enrolled and treated, 60% had HCV genotype 2a, 11% had cirrhosis, and 22% were over the aged 65 or older. Overall, 148 patients (97%) achieved SVR12. Of the 90 treatment-naïve patients, 88 (98%) achieved SVR12, and of the 63 previously treated patients, 60 (95%) achieved SVR12. The rate of SVR12 was 94% in patients with cirrhosis and in those aged 65 and older. No patients discontinued study treatment due to adverse events. The most common adverse events were nasopharyngitis, anaemia and headache. Twelve weeks of sofosbuvir and ribavirin resulted in high rates of SVR12 in treatment-naïve and previously treated patients with chronic genotype 2 HCV infection. The treatment was safe and well tolerated by patients, including the elderly and those with cirrhosis.

Keywords: Hepatitis C virus, HCV genotype 2, direct-acting antiviral agents, nucleotide polymerase inhibitor.

INTRODUCTION

Approximately two million people in Japan – nearly 2% of the population – are chronically infected with the hepatitis C virus (HCV) [1]. The population of patients with chronic HCV infection in Japan differs from that of other countries; patients are generally older, have more advanced liver disease and are more likely to have received previous treatment for HCV infection [2,3]. It is estimated that 15–30% of Japanese patients with HCV will develop serious complications, including liver cirrhosis, end-stage liver disease and hepatocellular carcinoma [4]. Although genotype 1 HCV is currently the most prevalent strain of the virus in Japan, genotype 2 HCV, which now accounts for up to 30% of infections, is rising in prevalence [5]. The current standard of care regimen for the treatment of chronic genotype 2 HCV infection in Japan is 24 weeks of pegylated interferon alpha (Peg-IFNα) and ribavirin (RBV) [6]. Although relatively high rates of SVR

Abbreviations: CI, confidence interval; GCP, Good Clinical Practice; HCV, hepatitis C virus; ICH, International Conference on Harmonization; Peg-IFNα, pegylated interferon alpha; PK, pharmacokinetics; RBV, ribavirin; SVR12, 12 weeks after therapy.

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Trial registration details: ClinicalTrials.gov number NCT01910636.

have been reported in clinical trials with this regimen (71–86%), the use of Peg-IFNα+RBV in an ageing population with progressive liver disease is limited by safety and tolerability issues. Moreover, a substantial number of patients have absolute or relative contraindications to interferon. As a result, many Japanese patients with chronic genotype 2 HCV infection have no available treatment options and are thus at risk for worsening of liver disease and complications of cirrhosis, including hepatocellular carcinoma.

Sofosbuvir (Gilead Sciences) is an oral nucleotide analogue inhibitor of the HCV-specific NS5B polymerase that has recently been approved in the United States and Europe for the treatment of chronic HCV infection [7]. The labelled use for patients with chronic genotype 2 HCV infection is sofosbuvir and RBV for 12 weeks. In phase 3 studies, 12 weeks of treatment with sofosbuvir plus RBV in patients infected with genotype 2 HCV resulted in rates of SVR12 of 97% in treatment-naïve patients, 93% in patients ineligible to receive interferon and 86–90% in previously treated patients [8–10].

We conducted a phase 3 trial to determine the efficacy and safety of 12 weeks of sofosbuvir and RBV in treatment-naïve and previously treated Japanese patients with chronic genotype 2 HCV infection with and without compensated cirrhosis.

METHODS

Patients

Patients were enrolled between 16 July 2013 and 30 September 2013 at 20 sites in Japan. Eligible patients were aged 20 years or older with a body weight of at least 40 kg. Patients were required to be chronically infected with genotype 2 HCV and with HCV RNA levels $\geq 10^4$ IU/mL at screening. Planned enrolment was for approximately 84 treatment-naïve and 50 previously treated patients. See Supplement for definitions of types of response to prior treatment.

Up to 40% of enrolled subjects in each group (i.e. treatment naive or treatment experienced) could have evidence of compensated cirrhosis at screening (Child-Pugh A). Cirrhosis was defined as liver biopsy showing a Metavir score of 4 or Ishak score ≥5 or a FibroScan score of >12.5 kPa. Patients were required to have ALT and AST ≤10 × upper limit of the normal range, platelet count ≥50 000 per μL, haemoglobin ≥11 g/dL for women and ≥12 g/dL for men and albumin ≥3 g/dL. There were no upper limits on age or body mass index. Similarly, no restriction was applied to white blood cell or absolute neutrophil count at screening.

Study design

In this multicenter, open-label trial, all patients received 12 weeks of treatment with 400 mg of sofosbuvir, administered orally once daily, and ribavirin (Copegus®, Chugai Pharmaceutical Co., Ltd, Tokyo, Japan), administered orally twice daily, with doses determined according to body weight (600 mg daily in patients with a body weight of ≤60 kg, 800 mg daily in patients weighing >60 and ≤80 kg, and 1000 mg daily in patients with a body weight of >80 kg).

In addition to the main study of efficacy and safety, sparse PK samples were collected from all patients over the course of the study for population PK analyses and all patients were eligible to participate in an optional substudy to determine the steady-state pharmacokinetics (PK) of sofosbuvir (and its predominant circulating metabolite GS-331007). The target enrolment per treatment group was approximately 15 patients. For the PK substudy, intensive serial pharmacokinetic samples were collected (samples obtained over 24 h postdose) at either the week 2 or week 4 treatment visits.

Study assessments

Screening assessments included serum HCV RNA levels and IL28B (rs12979860) genotyping, as well as standard laboratory and clinical tests. Serum HCV RNA was measured with the COBAS® TaqMan® HCV Test, version 2.0 for Use with the High Pure System (Roche Molecular Systems, West Sussex, UK), which has a lower limit of quantification (LLOQ) of 25 IU/mL. HCV genotype and subtype were determined at screening using the Siemens VERSANT HCV Genotype INNO-LiPA 2.0 assay.

On-treatment assessments included standard laboratory testing, serum HCV RNA, vital signs, electrocardiography and symptom-directed physical examinations. All adverse events were recorded and graded according to a standardized scale (see Supplementary Table S7).

NS5B amplification and deep sequencing was performed at DDL Diagnostics Laboratory (Rijswijk, The Netherlands) for all subjects who did not achieve SVR12. Deep sequencing of HCV NS5B was performed at the first virologic failure time point if a plasma/serum sample was available and HCV RNA was >1000 IU/mL, along with the respective baseline samples. Amino acid substitutions in NS5B in the samples collected at virologic failure were compared with the genotype 2 reference and the respective baseline sequence for each patient.

The population pharmacokinetic parameters for sofosbuvir and GS-331007 were computed for all subjects from concentration data from intensive and/or sparse samples using the previously established sofosbuvir and GS-331007 population PK models [11].

Statistical analysis

For treatment-naïve patients without cirrhosis, the SVR12 rate was compared to an adjusted historical SVR rate of 69%, using a two-sided exact one-sample binomial test. The historical control rate was calculated from the weighted average of historical SVR rates for noncirrhotic,

treatment-naïve Japanese patients with genotype 2 HCV infection receiving 24 weeks of Peg-IFNα+RBV (79% with a 10% discount applied due to the expected improvement in safety profile and shorter treatment duration – see Supplementary Table S2 for further details). We calculated that a sample size of 50 patients would provide 80% power to detect an 18% improvement in the SVR12 rate over the adjusted historical rate at a significance level of 0.05. For SVR12 rates for the overall population, for treatment-naïve patients with cirrhosis, and for previously treated patients, statistical hypothesis testing was not performed. For these outcomes, we calculated point estimates of SVR12 rates with two-sided 95% exact confidence interval using the binomial distribution (Clopper–Pearson method).

Study oversight

This trial was approved by the institutional review board or independent ethics committees at all participating sites and was conducted in accordance with local regulations and with recognized international scientific and ethical standards, including the International Conference on Harmonization (ICH) guideline for Good Clinical Practice (GCP) and the original principles embodied in the Declaration of Helsinki. The study was designed and conducted according to protocol by the sponsor (Gilead Sciences) in collaboration with the principal investigators. The sponsor collected the data, monitored study conduct and performed the statistical analyses. The manuscript was prepared by Gilead Sciences with input from all authors.

RESULTS

Baseline characteristics

Of the 188 patients who were initially screened, 153 (90 treatment-naïve and 63 previously treated patients) were enrolled and began treatment (Table S1 and Figure S1). The demographic and baseline clinical characteristics of the patients are provided in Table 1. Overall, the majority of patients were female (54%), and all were Japanese. The mean age was 57 years (ranging from 25 to 74 years) and 22% were aged 65 or older.

Previously treated patients were slightly older than the treatment-naïve patients, with a higher percentage of males, higher baseline viral load, with a higher prevalence of cirrho-

Table 1 Baseline Demographic Characteristics

Characteristic	Overall (N = 153)	Treatment naïve (n = 90)	Previously treated (n = 63)
Mean age, years (range)	57 (25, 74)	55 (25, 73)	60 (34, 74)
Mean BMI, kg/m^2 (range)	24 (16.5, 34)	24 (17, 34)	24 (16.5, 34)
Male, n (%)	70 (46)	33 (37)	37 (59)
Mean HCV RNA, $\log_{10}$ IU/mL ± SD	6.3 (0.84)	6.2 (0.92)	6.5 (0.66)
HCV RNA ≥5 $\log_{10}$ IU/mL, n (%)	140 (92)	78 (87)	62 (98)
HCV genotype, n (%)			
2a	92 (60)	52 (58%)	40 (63%)
2b	61 (40)	38 (42%)	23 (37%)
Cirrhosis, n (%)			
No	136 (89)	82 (91)	54 (86)
Yes	17 (11)	8 (9)	9 (14)
IL28B genotype, n (%)			
CC	121 (79)	73 (81)	48 (76)
CT	28 (18)	17 (19)	11 (17)
TT	4 (3)	0	4 (6)
Median baseline ALT, U/L (range)	34 (12, 412)	32 (12, 412)	36 (12, 232)
Baseline ALT >1.5 × ULN, n (%)	43 (28)	28 (31)	15 (24)
Interferon eligibility, n (%)*			
Interferon eligible	72 (80)	72 (80)	Not applicable
Interferon ineligible	5 (6)	5 (6)	Not applicable
Interferon unwilling	13 (14)	13 (14)	Not applicable
Response to prior HCV treatment, n (%)			
Nonresponse	15 (24)	Not applicable	15 (24)
Relapse/breakthrough	45 (71)	Not applicable	45 (71)
Interferon intolerant	3 (5)	Not applicable	3 (5)
Median eGFR, mL/min (range)	85 (51, 209)	86 (52, 175)	84 (51, 209)

*Interferon eligibility was determined by the site investigator based on whether or not, in their judgment, the patient had contraindications to interferon therapy.

sis and non-CC IL28B genotype. Overall, 11% of participating subjects had cirrhosis. The proportions of patients infected with genotype 2a and 2b HCV were 60% and 40%, respectively, which is similar to previous reports of HCV subtype distribution in the Japanese population [4]. Most (80%) of the treatment-naïve patients were considered eligible for interferon therapy, with 6% having contraindications to interferon therapy and 14% unwilling to receive this treatment. Most (71%) of the previously treated patients had experienced virologic breakthrough or relapse after previous treatment, with 24% reporting nonresponse to prior therapy.

Efficacy

Overall, 148 of the 153 patients (97%, 95% confidence interval [CI] 93–99%) achieved SVR12 (Table 2). By prior treatment history, 88 of the 90 treatment-naïve patients (98%, 95% CI, 92–100%) and 60 of the 63 previously treated patients (95%, 95% CI, 87–99%) achieved SVR12. Of the 82 treatment-naïve patients without cirrhosis, 80 (97%, 95% CI 91–100%) achieved SVR12, thus meeting the primary efficacy endpoint for this group of superiority to the adjusted historical control rate of 69% ($P < 0.001$). Of note, all eight treatment-naïve patients (100%) with cirrhosis and eight of the nine previously treated patients with cirrhosis (89%) achieved SVR12. Overall, 16 of the 17 patients with cirrhosis (94%, 95% CI 71–100%) achieved SVR12.

Patient responses according to baseline characteristics are shown in Supplementary Table S3. Rates of SVR12 were high in all subgroups of patients. Patients with characteristics historically associated with poor response to interferon-based treatment – non-CC IL28B genotype, high baseline viral load, elderly patients, cirrhosis – had rates of SVR12 similar to those in patients without these characteristics.

Relapse accounted for all cases of virologic failure; there were no patients with virologic breakthrough or nonresponse during treatment. Among all patients treated, 97% had HCV RNA <LLOQ by treatment week 2, and 100% achieved HCV RNA <LLOQ by treatment week 4. Overall, five patients experienced virologic relapse after the end of therapy: two (2%) treatment-naïve patients and three (5%) treatment-experienced patients. Four patients relapsed by post-treatment week 4, and one patient relapsed between post-treatment weeks 4 and 12. Characteristics of patients who relapsed are provided in Table S4. There were no consistent host or viral characteristics in the five subjects who relapsed; however, the number of virologic failures is too small for any conclusions to be drawn concerning predictors of virologic failure. No patient relapsed after post-treatment week 12. All 148 SVR12 patients (100%) also achieved SVR24.

Viral resistance testing

The NS5B region was deep sequenced in samples collected from the five relapsers at baseline and at the time of relapse. No S282T variant – known to be associated with reduced susceptibility to sofosbuvir – or any other nucleotide inhibitor resistance-associated variants were detected in any patient at relapse. Phenotypic analysis of the NS5B gene showed no change in susceptibility to either sofosbuvir or ribavirin.

Pharmacokinetics

Population pharmacokinetic analysis was performed to estimate the pharmacokinetics of sofosbuvir and its major circulating nucleoside metabolite, GS-331007. The mean (CV%) of steady-state AUC_{0-24} and C_{max} were 973 (31.2) ng*h/mL and 544 (33.6) ng/mL for sofosbuvir ($N = 45$), respectively, and 10 400 (27.2) ng h/mL and 818 (27.9) ng/mL for GS-331007 ($N = 153$), respectively. Within the Japanese study population, there were no clinically relevant differences in the pharmacokinetics of GS-331007 and sofosbuvir, based on age, sex, BMI, cirrhosis status, prior treatment experience or SVR12 outcome.

Safety

Overall, 73% of patients experienced at least one adverse event; however, the majority of patients experiencing

Table 2 Response during and after Treatment

Response	Overall ($N = 153$)	Treatment naïve ($n = 90$)	Previously treated ($n = 63$)
HCV RNA <LLOQ during treatment, *n* (%)*			
At week 2	148 (97%)	88 (98%)	60 (95%)
At week 4	153 (100%)	90 (100%)	63 (100%)
HCV RNA <LLOQ after end of treatment, *n* (%)			
SVR4	149 (97%)	89 (99%)	60 (95%)
SVR12	148 (97%)	88 (98%)	60 (95%)
95% confidence interval	92.5–99%	92–>99%	87–99%
On-treatment failure	0	0	0
Relapse, *n/n* (%)	5 (3%)	2 (2%)	3 (5%)

*LLOQ denotes lower limit of quantification, which is 25 IU/mL. SVR denotes sustained virologic response.

adverse events (84%) had only mild (grade 1) events. The most common treatment-emergent adverse events were nasopharyngitis (upper respiratory viral illness), anaemia, headache, malaise and pruritus (Table 3). No patient in the study discontinued treatment prematurely due to adverse events (or for any other reason). Twenty-two patients (14%) had adverse events that led to modification or interruption of a study drug; 20 patients had ribavirin dose reductions to manage anaemia, and one patient interrupted sofosbuvir and RBV for 1 day because of an event of nasopharyngitis. All but one of the 22 patients with modification or interruption of study drugs achieved SVR12. Two patients experienced treatment-emergent serious adverse events: one treatment-experienced 63-year-old woman had a worsening of anaemia for which she was hospitalized, and one treatment-naïve 36-year-old woman had a severe anaphylactic reaction to a bee sting. No patient experienced a life-threatening (grade 4) adverse event, and only three patients experienced severe (grade 3) events, two of which were deemed to be related to study treatment, the above-mentioned case of anaemia and one case of transient, ribavirin-associated hyperbilirubinaemia in a treatment-experienced 65-year-old man, which resolved during follow-up.

The overall rates of adverse events in younger (<65 years) and older (≥65 years) patients did not differ substantially (72% *vs* 76%, respectively), although there was a higher incidence of anaemia and pruritus in older patients (Table S5). The incidence and severity of adverse events in patients with and without cirrhosis at baseline were similar (Table S6).

Table 3 Discontinuations, Adverse Events and Laboratory Abnormalities by Age

Parameter	Overall (N = 153)
Discontinuation of any study drug due to adverse event	0
Serious adverse events	2 (1%)
Anaemia	1 (1%)
Anaphylactic reaction	1 (1%)
Any adverse event	112 (73%)
Common adverse events*	
Nasopharyngitis	45 (29%)
Anaemia	18 (12%)
Headache	15 (10%)
Malaise	11 (7%)
Pruritus	9 (6%)
Laboratory abnormalities, *n* (%)	
Decreased haemoglobin concentration	
<10 g/dL	19 (12%)
<8 g/dL	1 (1%)
Neutropenia (500–<750 per mm^3)	1 (1%)
Hyperglycaemia (>250–500 mg/dL)	3 (2%)
Hyperbilirubinaemia (>2.5–5.0 × ULN)	2 (1%)

ULN, upper limit of normal.
*Adverse events occurring in at least 5% of patients.

Overall, the mean change in haemoglobin from baseline to week 12 of treatment was −1.2 g/dL. For patients aged 65 and older, the mean change in haemoglobin was −1.7 g/dL, as compared with 1.0 g/dL in patients under the age of 65. Of all 153 patients enrolled and treated, 19 (12%) had at least one postbaseline haemoglobin value of <10.0 g/dL, and one (1%) had a postbaseline haemoglobin value of <8.5 g/dL. Two patients (1%) had grade 3 hyperbilirubinaemia; no grade 4 hyperbilirubinaemia occurred. One patient, who had grade 2 neutropenia at baseline, had transitory grade 3 neutropenia.

DISCUSSION

In this phase 3 trial, twelve weeks of treatment with sofosbuvir and RBV resulted in high rates of sustained virologic response (>95%) in treatment-naïve and previously treated Japanese patients with chronic genotype 2 HCV infection. Patients with host and viral characteristics that have historically been predictive of lower rates of SVR – older age, presence of cirrhosis, high viral load, non-CC IL28B alleles – had rates of SVR12 similar to patients without these characteristics. In patients who had been previously treated for HCV infection, the nature of the prior response was not associated with significant differences in rates of SVR following treatment with sofosbuvir and ribavirin; patients who had nonresponse to prior treatment had similar response rates as patients who had previously experienced relapse or viral breakthrough. No clear or consistent baseline predictors of treatment failure were evident among the five patients who relapsed after treatment.

The current standard-of-care treatment for Japanese patients with chronic genotype 2 HCV infection is 24 weeks of Peg-IFNα+RBV. Although patients who received this regimen in clinical trials achieved SVR12 rates ranging from 72% to 86%, these studies were restricted to patients <65 years of age [12,13]. However, the Japanese population chronically infected with genotype 2 HCV includes many patients with characteristics that make the use of interferon-based therapy problematic – older age, progressive liver disease, prior treatment experience and comorbid conditions such as diabetes and cardiovascular disease [14]. Moreover, many patients cannot receive interferon therapy due to relative or absolute contraindications. The interferon-free combination of sofosbuvir and ribavirin may represent a promising treatment option for these patients.

Given the characteristics of the patient population in Japan with HCV infection – generally older, and more likely to have advanced liver disease – safety and tolerability of therapeutic regimens is an important issue. In the present study, 22% of patients were aged 65 or older and 11% had cirrhosis. Analyses of safety data by age (<65 *vs*

≥65 years) showed increases in reported adverse events and laboratory abnormalities in older patients, but these differences did not present a barrier to treatment as no premature discontinuation of study treatment occurred in any patient. Analysis of safety data according to the presence or absence of cirrhosis did not indicate clinically important differences in safety or tolerability of the 12-week sofosbuvir plus ribavirin regimen.

Consistent with previous reports, the results of this study confirm the high barrier to resistance afforded by the sofosbuvir plus RBV treatment regimen. Rapid viral suppression was observed with all patients achieving HCV RNA undetectable status by week 4, with no virologic breakthrough observed during treatment in any of the 153 patients. The percentage of patients who relapsed after treatment was low (3%), and none of the subjects who relapsed had S282T or other nucleoside inhibitor resistance-associated variants. No change in susceptibility to sofosbuvir or ribavirin compared with the corresponding baseline or wild-type reference was observed at the relapse time point.

The main limitation of this study was the lack of a control arm to allow direct comparison with interferon-based regimens. Several considerations guided our choice of an uncontrolled study design. Adding an interferon-based control arm would have required exclusion of patients who were ineligible to receive or intolerant of interferon – an important and substantial proportion of patients – as well as previously treated patients, for whom further interferon treatment is not an option. Moreover, given that Peg-IFNα is administered by subcutaneous injection, blinding of treatment arms would not have been possible.

In conclusion, treatment with the all-oral, interferon-free combination of sofosbuvir and RBV resulted in high rates of sustained virologic response in both treatment-naïve and previously treated Japanese patients with chronic genotype 2 HCV infection. The degree of antiviral efficacy coupled with a favourable safety and tolerability profile, including patients with cirrhosis and those aged 65 and older, suggest that this combination may fill an important unmet medical need in Japan.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Patient disposition.

Table S1. Reasons for screen failure.

Table S2. Calculation of the adjusted historical control rate.

Table S3. SVR12 by subgroup.

Table S4. Characteristics of patients who relapsed.

Table S5. Common adverse events by age group.

Table S6. Common adverse events by cirrhosis status.

Table S7. Gilead sciences grading scale for severity of adverse events and laboratory abnormalities.

Hepatology Research

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Review Article

Mitochondrial reactive oxygen species as a mystery voice in hepatitis C

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There are several lines of evidence suggesting that oxidative stress is present in hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression. The main production site of reactive oxygen species (ROS) is assumed to be mitochondria, which concept is supported by evidence that hepatitis C virus (HCV) core protein is directly associated with them. The detoxification of ROS also is an important function of the cellular redox homeostasis system. These results draw our attention to how HCV-induced mitochondrial ROS production is beyond redox regulation and affects the disease progression and development of hepatocellular carcinoma (HCC) in chronic hepatitis C. On the other hand, HCV-related chronic liver diseases are characterized by metabolic alterations such as insulin resistance, hepatic steatosis and/or iron accumulation in the liver. These metabolic disorders also are relevant to the development of HCC in HCV-related chronic liver diseases. Here, we review the mechanisms by which HCV increases mitochondrial ROS production and offer new insights as to how mitochondrial ROS are linked to metabolic disorders such as insulin resistance, hepatic steatosis and hepatic iron accumulation that are observed in HCV-related chronic liver diseases.

Key words: calcium signaling, mitochondrial electron transport, mitophagy, hepcidin, insulin resistance, iron metabolism

INTRODUCTION

APPROXIMATELY 170 MILLION people worldwide are infected with hepatitis C virus (HCV).[1] HCV infection often remains asymptomatic, but can lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC).[2] Although the mechanisms of its pathogenesis are incompletely understood, there are several lines of evidence suggesting that oxidative stress is present in hepatitis C to a greater degree than in other inflammatory liver diseases and is closely related to disease progression.[3–6] Previous *in vitro* and *in vivo* studies have shown that HCV core protein induces the production of reactive oxygen species (ROS)[7–9] and that mitochondrial electron transport inhibition by HCV core protein is associated with ROS production.[10,11] These results draw our attention to how HCV-induced mitochondrial injury contributes to disease progression and hepatocarcinogenesis in hepatitis C.

On the other hand, HCV-related chronic liver diseases are characterized by metabolic alterations such as insulin resistance,[12–14] hepatic steatosis[15,16] and/or iron accumulation in the liver.[3,17] These metabolic disorders also are relevant to the development of HCC in HCV-related chronic liver diseases.[18–21] The present review highlights the mechanisms underlying the production of mitochondrial ROS by HCV and the metabolic disorders induced by mitochondrial dysfunction, and discuss how mitochondrial ROS contribute to the disease progression and hepatocarcinogenesis in hepatitis C.

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MITOCHONDRIAL ROS PRODUCTION

Mitochondrial electron transport and ROS production

THE MITOCHONDRIAL ELECTRON transport system consists of several multi-polypeptide protein complexes (I–V) embedded in the inner mitochondrial membrane that receive electrons from reducing equivalents (i.e. nicotinamide adenine dinucleotide and

$FADH_2$) generated by dehydrogenases (e.g. pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, acyl-coenzyme A dehydrogenase). These electrons flow through complex I, the ubiquinone cycle (Q/QH_2), complex III, cytochrome c, complex IV, and to the final acceptor O_2 to form H_2O. Electron flow through complexes I, III and IV results in the pumping of protons to the outer surface of the inner membrane, establishing a membrane potential that is used by adenosine triphosphate synthetase to drive the re-phosphorylation of adenine dinucleotide phosphate. Several of the redox couples within the electron transport chain transfer single rather than two electrons and are therefore susceptible to leaking electrons directly to surrounding O_2 to form the free-radical superoxide ($O_2\bullet^-$). The detoxification of ROS is an important function of the cellular redox homeostasis system. Cells rapidly convert $O_2\bullet^-$ into the two-electron non-radical hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD). H_2O_2 in turn can be further reduced to H_2O in the mitochondrial matrix by glutathione (GSH) or the thioredoxin/peroxiredoxin systems, or can freely diffuse out of the mitochondria where it again is buffered by GSH.[22]

Interaction of HCV core protein with mitochondria and mitochondrial ROS production

Hepatitis C virus core protein has been shown to directly associate with mitochondria. While the initial reports showed that HCV core protein associated exclusively with the mitochondrial outer membrane via a C-terminal motif,[10,23] a recent study using electronic microscopy suggests that HCV core protein is also associated with the mitochondrial inner membrane.[24] Importantly, Schwer *et al.* have demonstrated that core protein associates with the mitochondria-associated membrane (MAM) fraction, a point of close contact between the endoplasmic reticulum (ER) and mitochondrion.[23] In addition, biochemical evidence suggests that the interaction also takes place in the context of productively replicative HCV in cell culture (HCVcc),[25] even though subcellular analysis using confocal microscopy did not confirm a direct interaction of the HCV core with mitochondria in HCVcc infected Huh7.5 cells.[26] Direct interaction of HCV core protein with mitochondria potentially modifies mitochondrial ROS production and scavenging, which subsequently induce oxidative stress. The effects of HCV on ROS production and scavenging are summarized in Table 1.[27] When mitochondrial electron transport activity is inhibited by HCV core protein,[10,28] electrons are likely to leak from the electron transport chain transfer, accelerating mitochondrial $O_2\bullet^-$ production and/or H_2O_2 emission. Induction of mitochondrial and/or cellular antioxidant enzymes concomitantly with ROS production may be explained by antioxidant defense mechanisms rather than direct induction of antioxidant enzymes by HCV, even though HCV core and non-structural proteins have been reported to lead to different effects on cellular

Table 1 Effects of HCV on ROS production or scavenging *in vitro* and *in vivo*

Effect on ROS production or scavenging	*In vitro* and *in vivo* HCV models	References
Inhibition of mitochondrial electron transport	Structural gene transgenic mice, core gene transgenic mice, full genomic replicon cells	10, 28
Oxidation of glutathione pool	Structural gene transgenic mice, core- and non-structural protein-expressing cell lines	10, 29
Induction of mitochondrial antioxidant enzymes	Non-structural protein-expressing cell lines	29
Inhibition of gastrointestinal-glutathione peroxidase	Subgenomic replicon cells	30
Induction of glutathione peroxidase	Core-expressing cell line, subgenomic replicon cells	8, 30
Oxidation of the thioredoxin pool	Core-expressing cell line	29
Increase in lipid peroxidation	Core-expressing cell line, core gene transgenic mice	8, 31
Induction of metallothionein	Core-expressing cell line	8, 32
Cytoplasmic ROS production by NADPH oxidase	Core-expressing cell line, subgenomic replicon cells, full genomic replicon cells, HCV-infected Huh7 cells (JFH)	33, 34

HCV, hepatitis C virus; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

antioxidant defenses.[29] Thus, one of the major sources for intracellular ROS production by core protein is the mitochondrion, even though the core is also involved in ROS production at the plasma membrane by activating nicotinamide adenine dinucleotide phosphate oxidase 4.[33,34]

Modulated calcium signaling by HCV and mitochondrial ROS production

The close physical association between the ER and mitochondria mediated by MAM results in Ca^{2+} microdomains at contact points that facilitate efficient Ca^{2+} transmission from the ER to mitochondria.[35] Although sufficient intra-organelle Ca^{2+} concentrations are required to stimulate metabolism by activating enzymes critical for maintenance of the tricarboxylic acid (TCA) cycle,[36] prolonged increases of Ca^{2+} can, in turn, interfere with the activity of these enzymes. The TCA cycle activity affects the electron transport chain activity, which in turn affects the mitochondrial membrane potential ($\Delta\Psi$). Thus, increased Ca^{2+} influx to mitochondria induces a substrate imbalance of the TCA cycle that leads to the generation of mitochondrial ROS, probably through the inhibition of electron transport chain activity. There are several lines of evidence indicating that HCV increases mitochondrial ROS production by modulating calcium signaling.[37–39] The HCV NS5A protein is reported to cause a disturbance of intracellular Ca^{2+} signaling, which triggers mitochondrial ROS production.[37] As shown in Figure 1, HCV core protein also enhances mitochondrial Ca^{2+} uptake in response to ER Ca^{2+} release through activation of the mitochondrial Ca^{2+} uniporter, which leads to increased mitochondrial ROS production.[38,39] Pharmacological inhibition of ER–mitochondrial Ca^{2+} fluxes, but not ROS scavengers, has been shown to normalize all aberrant effects induced by HCV: normalization of the electron transport chain complex I activity, restoration of mitochondrial $\Delta\Psi$ and normalization of ROS concentrations. More importantly, the time course and titration of HCV polyprotein expression suggest that mitochondrial Ca^{2+} uptake is the earliest of these above events induced by HCV.[39] Thus, mitochondrial Ca^{2+} uptake may be the initial event associated with mitochondrial dysfunction induced by HCV and may, in turn, trigger complex I inhibition, loss of mitochondrial $\Delta\Psi$ and ROS production. All these effects could be counteracted by intracellular Ca^{2+} chelation, suggesting that control of mitochondrial Ca^{2+} uptake may be useful as a new therapeutic intervention.

MITOCHONDRIAL QUALITY CONTROL

AS MENTIONED ABOVE, the detoxification of ROS is an important function of the cellular redox homeostasis system. Under resting cellular conditions, the intracellular redox environment is in a relatively reduced state.[40] Therefore, the next question is how HCV core-induced mitochondrial ROS production and the subsequent oxidative stress persist in spite of the presence of ROS-detoxifying agents such as MnSOD and/or GSH or the thioredoxin/peroxiredoxin systems. There are several lines of evidence indicating that mitochondrial injury is present in patients with chronic hepatitis C[4] and transgenic mice expressing the HCV core protein.[19] Although it remains unknown whether damaged mitochondria behave as an active ROS source, they are assumed to have less ROS-detoxifying activity than intact mitochondria. In mammalian cells, the autophagy-dependent degradation of mitochondria (mitophagy) is thought to maintain mitochondrial quality by eliminating damaged mitochondria.[41,42] Indeed, mitophagy plays an essential role in reducing mitochondrial ROS production and mitochondrial DNA mutations in yeast.[43] Mitochondrial membrane depolarization precedes the induction of mitophagy,[44] which is selectively controlled by a variety of proteins in mammalian cells, including phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) and the E3 ubiquitin ligase Parkin.[41,45] PINK1 facilitates Parkin targeting to the depolarized mitochondria[45] and, although Parkin ubiquitinates a broad range of mitochondrial outer membrane proteins,[45] it remains unclear how Parkin enables damaged mitochondria to be recognized by the autophagosome. We recently found that HCV core protein suppresses mitophagy by inhibiting the translocation of Parkin to the mitochondria via a direct interaction with it (Yuichi Hara, unpubl. data, 2013). Considering that oxidative stress and/or hepatocellular mitochondrial alterations are present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases[3–6] and that mitophagy is important for maintaining mitochondrial quality by eliminating damaged mitochondria, our finding that HCV core protein suppresses mitophagy may in part explain the pathophysiology of chronic hepatitis C. However, in contrast to our results, Siddiqui *et al.* have shown that HCV induces the mitochondrial translocation of Parkin and subsequent mitophagy.[46] In addition, their results indicated that the HCV-mediated decline of mitochondrial complex I enzyme activity was rescued by chemical inhibition of mitophagy or by Parkin silenc-

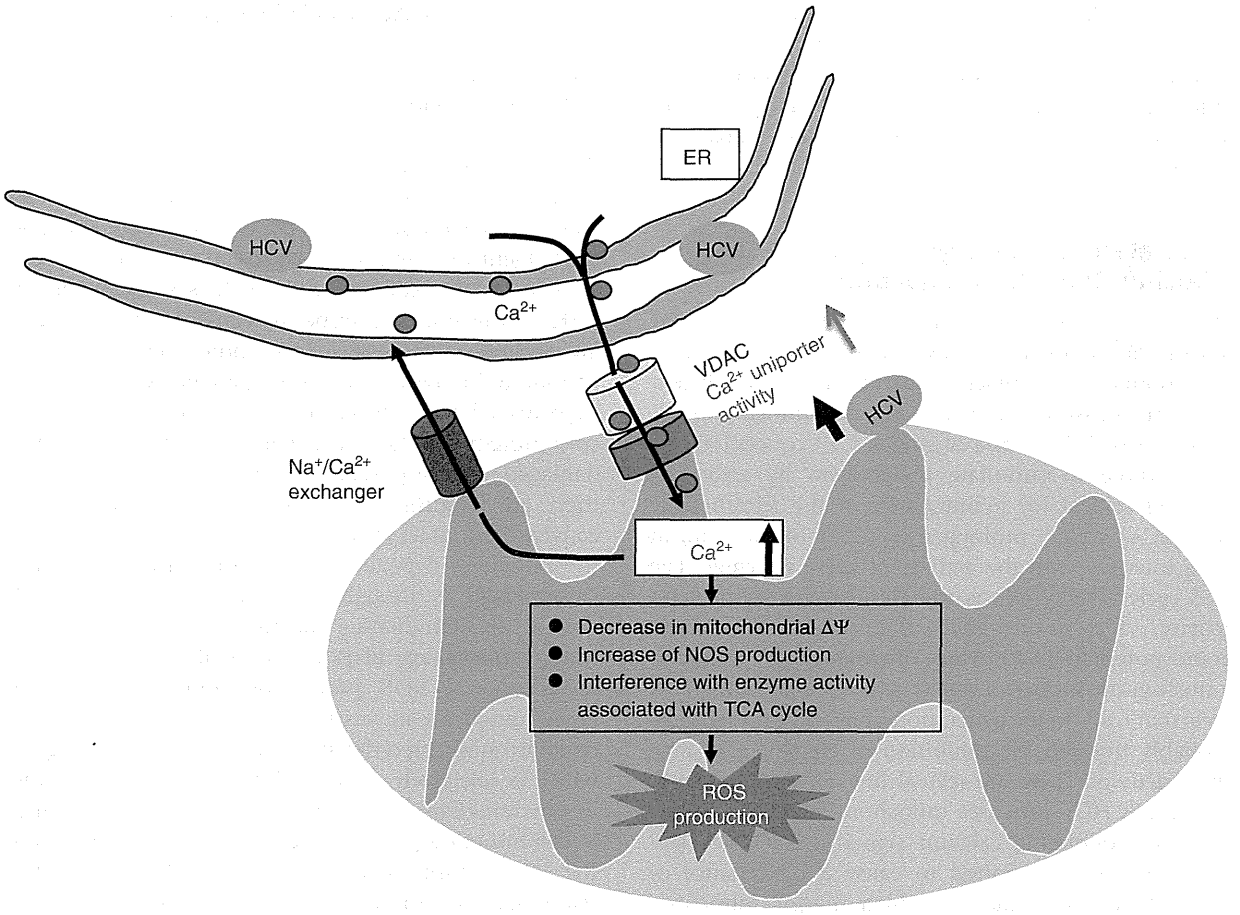

Figure 1 Schematic diagram depicting hepatitis C virus (HCV)-related calcium transfer from the endoplasmic reticulum (ER) to the mitochondria in the mitochondria-associated membrane (MAM) fraction. HCV core protein enhances mitochondrial Ca^{2+} uptake in response to ER Ca^{2+} release through activation of the mitochondrial Ca^{2+} uniporter, which leads to increased mitochondrial ROS production The voltage-dependent anion channel (VDAC) is the major component of the mitochondrial permeability transition (MPT) pore. It is assumed that there is spatial proximity of the complexes responsible for ER Ca^{2+} release, mitochondrial Ca^{2+} uptake and the MPT pore.

ing, and suggested that induction of mitophagy by HCV may significantly contribute to the mitochondrial injury associated with chronic hepatitis C. Thus, it still remains a matter of debate as to whether HCV induces or suppresses mitophagy and how oxidative stress persists in HCV infection.

METABOLIC ALTERATIONS BY MITOCHONDRIAL ROS

Insulin resistance

TYPE 2 DIABETES mellitus is one of the important extrahepatic manifestations associated with chronic HCV infection.[47,48] The final common pathway responsible for the development of type 2 diabetes mellitus is the failure of the pancreatic β-cells to compensate for insulin resistance. Although the molecular mechanisms by which HCV promotes insulin resistance have not been fully elucidated, there are several lines of evidence suggesting that HCV directly induces insulin resistance.[49–51] Insulin receptor substrate (IRS)1 and IRS2 are normally expressed in hepatocytes and central molecules of the hepatic insulin signal cascade. HCV core protein is reported to upregulate suppressor cytokine signal (SOCS)3 and cause ubiquitination of IRS1 and IRS2, leading to their proteosomal degradation.[50] SOCS3 also suppresses phosphorylation of tyrosine within IRS1.[52,53] Inhibition of tyrosine phos-

phorylation within IRS1 due to a high level of tumor necrosis (TNF)-α which leads to suppression of downstream insulin signals has been shown in HCV core transgenic mice.[49]

What roles do mitochondrial ROS play in HCV-induced insulin resistance? TNF-α phosphorylates Ser^{307} of IRS1 through serine (Ser) kinases such as c-Jun N-terminal kinase (JNK), which disrupts the interaction between the catalytic domains of the insulin receptors and the phosphotyrosine-binding domain of IRS1,[54,55] even though there is a contrary report that Ser^{307} promotes insulin sensitivity in mice.[56] The intracellular redox condition is a master regulator of phosphorylation/dephosphorylation events due to the presence of redox-sensing cystein (Cys) residues within nearly all classes of protein phosphatase enzymes.[57] In general, phosphatase activity is depressed in response to an oxidative shift in the redox environment, thus leading to a concomitant increase in kinase activity either via direct oxidant-induced activation or secondary to phosphatase inactivation.[57] Inactivation of protein tyrosine phosphatases is mediated via the oxidation of a conserved redox sensitive Cys residue within their catalytic sites, which must be in the reduced state as the thiol (-SH) to form a cysteinyl-phosphate intermediate during hydrolysis.[58] Thus, research conducted over the past several years has established a role for the activation of stress sensitive Ser/threonine (Thr) kinases and their subsequent phosphorylation of inhibitory Ser/Thr residues within the insulin receptor and IRS1/2 as a potential mechanism of insulin resistance.[59] In agreement with the evidence suggesting a central role for ROS in the development of insulin resistance,[60] HCV core-induced mitochondrial ROS production is presumed to induce insulin resistance through activation of Ser/Thr kinases such as JNK1 and subsequent inhibition of tyrosine phosphorylation within IRS1 (Fig. 2). Hepatic insulin resistance induces suppressed insulin clearance as well as increased insulin secretion from pancreatic β-cells, which leads to hyperinsulinemia and represses whole-body insulin sensitivity.[61]

Hepatic steatosis

Hepatic steatosis is also one of the pathophysiological features of HCV-associated chronic liver disease.[15,16] It is characterized by the cytoplasmic accumulation of lipid droplets, mainly composed of triglyceride and cholesterol ester. The composition of triglycerides in the liver is uniquely and significantly enriched in carbon monosaturated (C18:1) fatty acids in chronic hepatitis C,[62] which is distinct from what occurs in obese patients. The mechanisms underlying HCV-related steatosis are diverse: decreased lipoprotein secretion from hepatocytes, increased synthesis of fatty acids, decreased fatty acid oxidation and increased fatty acid uptake by hepatocytes. The HCV core protein has been demonstrated to inhibit microsomal transfer protein activity[63] and to upregulate transcriptional activity of sterol regulatory element-binding protein 1, a transcription factor involved in lipid synthesis.[64] These observations underscore the importance of the core as a direct and principal regulator of HCV-associated steatosis. On the other hand, decreased fatty acid oxidation and increased fatty acid uptake are related to mitochondrial dysfunction and hyperinsulinemia, respectively. Indeed, we previously demonstrated impaired mitochondrial fatty acid oxidation concomitant with increased ROS production in iron-overloaded transgenic mice expressing the HCV polyprotein.[65] Hyperinsulinemia derived from insulin resistance inhibits lipolysis in the liver and increases fatty acid uptake by hepatocytes. As described above, mitochondrial ROS production is presumed to induce insulin resistance. Thus, inhibited fatty acid oxidation and increased fatty acid uptake are potentially related to mitochondrial ROS production induced by the core protein.

Hepatic iron accumulation

Elevated iron-related serum markers and increased hepatic iron accumulation are relatively common and correlate with the severity of hepatic inflammation and fibrosis in patients with chronic hepatitis C. Excess divalent iron can be highly toxic, mainly via the Fenton reaction producing hydroxyl radicals.[66] This is particularly relevant for chronic hepatitis C, in which oxidative stress has been proposed as a major mechanism of liver injury. Oxidative stress and increased iron levels strongly favor DNA damage, genetic instability and tumorigenesis. Indeed, a significant correlation between 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidatively generated DNA damage,[67] and hepatic iron excess has been shown in patients with chronic hepatitis C.[68] We showed that transgenic mice expressing the HCV polyprotein fed an excess-iron diet developed HCC through hepatic accumulation of 8-OHdG.[65]

Here, we discuss the mechanisms by which hepatic iron accumulates in chronic hepatitis C, focusing on the relationship between HCV-induced ROS production and iron metabolic disorder. Systemic iron homeostasis is mainly regulated both by intestinal absorption and macrophage recycling of iron from hemoglobin because there is no efficient pathway for iron excretion.[69]

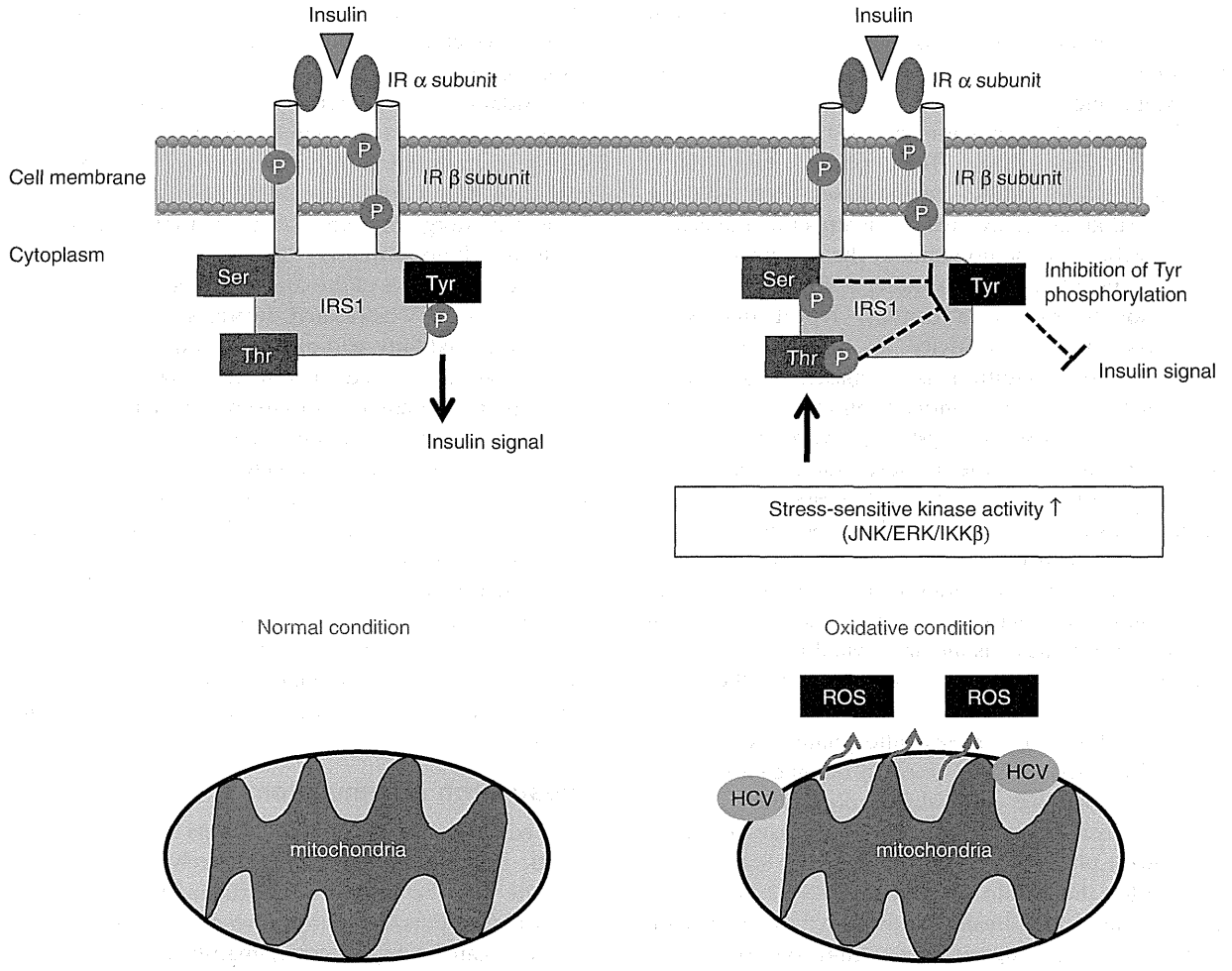

Figure 2 Schematic diagram depicting the presumed mechanism underlying the inhibitory effect on tyrosine phosphorylation within insulin receptor substrate 1 (IRS1). Hepatitis C virus (HCV) core-induced mitochondrial reactive oxygen species (ROS) production is presumed to induce insulin resistance through activation of Ser/Thr kinases such as c-Jun N-terminal kinase-1 (JNK1) and subsequent inhibition of tyrosine phosphorylation within IRS1. IR, insulin receptor; Ser, serine; Thr, threonine; Tyr, tyrosine; ERK, extracellular signal-regulated kinase; IKKβ, inhibitory-κβ kinase β.

Hepcidin, which was originally isolated from human serum and urine as a peptide with antimicrobial activity,[70] is a hormone exclusively synthesized in the liver and a soluble regulator that acts to attenuate both intestinal iron absorption and iron release from reticuloendothelial macrophages.[71] Hepatic mRNA levels[72] and the 25 amino acid bioactive hepcidin levels in serum[73] are lower in chronic hepatitis C than in chronic hepatitis B or controls, despite a significant correlation between hepcidin and serum ferritin or the histological iron score. Thus, the relatively decreased synthesis of hepcidin in chronic hepatitis C contrasts with the absolute deficit or lack of hepcidin synthesis observed in hereditary hemochromatosis. The detailed mechanisms underlying the transcriptional regulation of hepcidin are discussed elsewhere. Interestingly, alcohol metabolism-mediated ROS were shown to suppress hepcidin transcription via CCAAT/enhancer-binding protein α (C/EBPα).[74] In parallel with these results, we found that hepcidin promoter activity and the DNA binding activity of C/EBPα were downregulated concomitant with increased expression of C/EBP homology protein (CHOP), an inhibitor of C/EBP DNA binding activity, and with increased levels of mitochondrial ROS in