Insulin resistance and HCV: a case-control study of non-obese, non-alcoholic, and non-steatotic hepatitis virus carriers with persistently normal serum aminotransferase

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Abbreviations: ALT, alanine aminotransferase; APRI, aspartate aminotransferase-to-platelet count ratio index; AST, aspartate aminotransferase; BMI, body mass index; FPG, fasting plasma glucose; γGT, γ-glutamyltransferase; HBV, hepatitis B virus; HCV, hepatitis C virus; HOMA, homeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; IR, insulin resistance; IRI, immunoreactive insulin; NASH, nonalcoholic steatohepatitis; PNALT, persistently normal serum alanine aminotransferase; TG, triglyceride; TNF, tumor necrosis factor; US, ultrasonography.

Abstract

Background/Aims: Recent studies using transgenic mouse models have demonstrated that the presence of hepatitis C virus (HCV) singularly induces insulin resistance (IR). When evaluated in humans, the exclusion of other factors influencing IR, such as obesity, alcohol intake, hepatic inflammation, and steatosis is needed, but there have been few studies done to these ends. Therefore, we aimed to explore the singular effects of HCV on glucose metabolism through analysis of HCV carriers with persistently normal serum aminotransferase.

Methods: Non-obese, non-diabetic, and non-alcoholic HCV carriers (n = 30) were enrolled with 30 HBV carriers matched by age, gender, body mass index, and waist-to-hip ratio. All patients maintained normal serum aminotransferase (< 30 U/L), hyaluronic acid (< 50 ng/mL), and platelet count (> 150 x 10^3/μL) for more than 5 years without additional treatments, and had no signs of steatosis. We then compared fasting plasma glucose, serum insulin and adiponectin, and homeostasis model assessment of IR (HOMA-IR) and HOMA-β indices between the groups.

Results: There were no significant differences in insulin resistance/secretion-associated markers or serum adiponectin. Multivariate analysis demonstrated that the presence of HCV was not an independent predictor of IR. HOMA-IR was strongly correlated with waist circumferences and serum γ-glutamyltransferase in HCV carriers, but not with serum aminotransferase, high-sensitivity C-reactive protein, hyaluronic acid, or HCV core antigen.

Conclusions: These results suggest that the presence of HCV alone does not affect IR. Coexistence of hepatitis, steatosis and/or fibrosis may be important to the pathogenesis of IR induced by chronic HCV infection.

Introduction

There have been several epidemiological studies that have demonstrated a close association between chronic hepatitis C virus (HCV) infection and diabetes mellitus (1-3), and insulin resistance (IR) is the main feature of impaired glucose metabolism caused by HCV infection (4). Although the mechanism of IR has not been fully elucidated, increased triglyceride (TG) (5, 6) and/or iron accumulation (7-9) in the liver and advanced hepatic fibrosis (10-12) are both thought to be contributors. The homeostasis model assessment of IR (HOMA-IR) in the early stages of chronic hepatitis C patients are reported to be greater than those in healthy volunteers matched by age, gender, body mass index (BMI), and waist-to-hip ratio (13), suggesting the presence of an HCV-specific mechanism of IR independently of progression of hepatic fibrosis.

The possibility that HCV and/or the core protein itself can induce HCV-specific IR is based on several findings. First, insulin receptor substrate-1 and -2, which are central molecules in the insulin signaling cascade, are down-regulated in the livers of HCV core protein transgenic mice and in the core protein-transfected human hepatoma cell lines (14). Second, transgenic mice constitutively expressing core protein in livers exhibited marked hyperinsulinemia and IR, which were ameliorated by inhibition of the tumor necrosis factor (TNF)-α pathway (15). Finally, HCV eradication by interferon therapy improved IR in patients with chronic hepatitis C (16). However, since factors such as obesity, hepatocyte injury, hepatic inflammation, and steatosis, have been shown to affect the onset of IR (17-19), they need to be excluded when the sole effect of HCV-specific IR in humans is discussed. As far as we know, there are scarce few studies that assess IR in chronically HCV-infected patients after careful adjustment for these factors, so we sought to determine the contribution of HCV alone on the pathogenesis of IR by comparing the insulin resistance/secretion-related parameters between HCV and hepatitis B virus (HBV) carriers with persistently normal serum alanine aminotransferase (ALT) (< 30 U/L) (PNALT) (20). Before comparison, several other known factors that influence IR, such as obesity, alcohol intake (21), hepatic steatosis, and fibrosis, were excluded, and both groups were carefully matched by age, gender, BMI, and waist-to-hip ratio. IR was not found in non-obese, non-diabetic, non-alcoholic, and non-steatotic HCV carriers with PNALT, implying that the presence of HCV per se cannot induce IR. In other words, our results suggest that various other hepatic abnormalities caused by HCV infection, including hepatitis, steatosis, or fibrosis, are likely necessary for the pathogenesis of IR in chronically HCV-infected patients.

Patients and methods

Patients

Patients selection was carried out as shown in Fig. 1. HCV and HBV carriers with PNALT were defined as patients who were positive for HCV-RNA and HBV surface antigen in sera, respectively, but who had normal serum ALT (< 30 U/L), hyaluronic acid (< 50 ng/mL), and platelet count (> 150 x 10^3/μL) for more than 5 years without any treatments. Serum ALT and platelet count had been measured at least every 3 months, and serum hyaluronic acid had been measured every 6 months prior to the study. Patients who had previously been administered interferon injections and/or antiviral or hepatoprotective drugs were excluded. All HBV carriers were negative for HBe antigen and had viral loads of less than 1000 copies/mL. To minimize any other factors affecting IR, additional criteria have been strictly upheld for more than 5 years: (1) BMI < 25 kg/m^2, as calculated every 3 months, (2) alcohol consumption < 20 g/day, (3) fasting plasma glucose (FPG) < 7 μmol/mL or taking no insulin or oral hypoglycemic drugs, (4) the absence of steatosis, advanced fibrosis, and cirrhosis, as detected by repeated abdominal ultrasonography (US) every 6 months, (5) the absence of ongoing treatment with corticosteroids or any other medication known to affect glucose tolerance or insulin secretion, and (6) the absence of other concomitant diseases such as human immunodeficiency virus infection, hereditary hemochromatosis, pancreatitis, renal failure, or neoplasia. Subsequently, both groups were carefully matched by age, gender, BMI, and waist-to-hip ratio, and non-obese, non-alcoholic, non-diabetic, and non-steatotic HCV (n = 30) and HBV (n = 30) carriers with PNALT were enrolled in this study (Fig. 1).

Informed consent, in writing, was obtained from all patients. Body height, weight, and waist and hip circumferences were measured in the fasting state by hospital staff unaware of the patients' medical information. Any underlying diseases, medical interventions, past medical history, and family history of diabetes were also recorded. Patients were considered hypertensive if their systolic/diastolic pressure was greater than 140/90 mmHg, or if they were taking anti-hypertensive drugs. Patients were considered to have hyperlipidemia if their fasting serum cholesterol and TG were equal to or higher than 220 mg/dL and 150 mg/dL, respectively, or if they were taking lipid-lowering drugs (5, 22). The presence of metabolic syndrome was judged according to the new definition released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome (23).

Laboratory examination

Venous blood samples were drawn from patients after an overnight fast. Serum insulin was determined by the radioimmunoassay method, and other data were measured by standard methods using a conventional automated analyzer. HOMA-IR was calculated using the following equation: [FPG (μmol/mL) x immunoreactive insulin (IRI) (μU/mL)]/22.5. A HOMA-IR of greater than 1.73 was considered indicative of the presence of IR, which was estimated using the M value from the euglycemic-hyperinsulinemic clamp method in the Japanese population (24). HOMA-β, a parameter reflecting the insulin secretion ability of pancreatic β-cells, was calculated as follows: [360 x IRI (μU/mL)]/[FPG (μmol/mL)/0.0555 - 63]. Serum adiponectin was measured by means of an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). Serum hyaluronic acid and high-sensitivity C-reactive protein (hsCRP) were examined by latex agglutination-turbidimetric immunoassay (Fujirebio Inc, Tokyo, Japan) and latex nephelometry (Dede Behring, Deerfield, IL, USA), respectively. Serum amount of HCV core antigen was determined by a chemiluminescence enzyme immunoassay method (Eiken Chemical Co. Ltd, Tokyo, Japan). Serum aspartate aminotransferase (AST)-to-platelet count ratio index (APRI), a well-known indicator of hepatic fibrosis (25, 26), was calculated as follows: AST (U/L)/33 (upper limit of normal range of AST)/platelet count (x 10^3/μL) x 100.

Imaging examination

Each patient underwent abdominal US (Hitachi model EUB-525 equipped with a 3.5 MHz convex-type transducer, Hitachi, Japan) in a fasting state. The presence of hepatic steatosis was assessed according to findings such as hepatorenal contrast, blurring of vascular walls, and profound attenuation of the diaphragm (5, 27). The presence of advanced fibrosis or cirrhosis was evaluated by the presence of splenomegaly, hypertrophy of left or caudal lobes, and surface irregularity (28). Images were evaluated and judged by an independent ultrasonographer uninformed about the clinical data of the patients.

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration and was approved by the hospital's human ethics committee.

Statistical analysis

Results are expressed as mean $\pm$ SD or median and range (in parenthesis). Statistical analyses were performed using SPSS software 11.5J for Windows (SPSS Inc., Chicago,

IL, USA). Comparisons between the two groups were made using Fisher's exact probability test for categorical variables and the Student's *t* test for continuous variables. All *P* values were based on a two-sided test of statistical significance. Correlation coefficients were calculated using Spearman's rank correlation analysis. A *P* value of less than 0.05 was considered statistically significant.

Results

Clinical features and biochemical parameters of hepatitis virus carriers with PNALT

The clinical features of both groups are shown in Table 1. The prevalence of hypertension and hyperlipidemia was similar between the groups, and no participants had a family history of diabetes or fulfilled the Japanese criteria for metabolic syndrome. There were no significant differences in age, gender, BMI, waist-to-hip ratio, platelet count, hsCRP, serum AST or ALT, γ-glutamyltransferase (γGT), hyaluronic acid, or APRI. Although it has been reported that serum cholesterol and ferritin are elevated in patients with chronic hepatitis C compared to those with chronic hepatitis B (29, 30), these factors were similar between the groups. HCV genotype was 1b in all patients, and overall HCV core antigen concentrations were variable.

Comparison of insulin resistance/secretion-related parameters between HCV and HBV carriers with PNALT

Next, serum insulin and adiponectin, as well as the parameters related to insulin resistance (HOMA-IR) and secretion (HOMA-β), were compared between HCV and HBV carriers. We found no significant differences in FPG (5.27 ± 0.72 vs. 5.33 ± 0.78 μmol/mL, $P = 0.83$), serum IRI (5.1 ± 2.8 vs. 7.9 ± 6.6 μU/mL, $P = 0.11$), or adiponectin (16.5 ± 6.3 vs. 13.9 ± 5.9 μg/mL, $P = 0.21$). HOMA-IR in HCV carriers were similar to those in HBV carriers (1.2 ± 0.8 vs. 1.8 ± 1.3, $P = 0.12$), and HOMA-β did not differ between the two groups (59 ± 31 vs. 123 ± 84, $P = 0.18$) as well. These results demonstrate that HCV-specific IR does not occur in non-obese non-steatotic HCV carriers with PNALT.

Multivariate analysis

Multivariate analysis was performed to investigate the contribution of HCV infection to IR. There were no independent predictors of IR, including the presence of HCV.

Correlation between clinical parameters and insulin resistance/secretion markers in HCV carriers with PNALT

To explore the clinical indicators associated with insulin resistance/secretion in the HCV carriers with PNALT, correlations between several clinical parameters with HOMA-IR, serum IRI/adiponectin, and HOMA-β were analyzed. HOMA-IR was strongly correlated with waist circumference ($r = 0.580$, $P = 0.006$) and serum γGT ($r = 0.554$, $P = 0.004$) and TG ($r = 0.529$, $P = 0.007$). HOMA-IR was also associated with hip circumference ($r = 0.496$, $P = 0.022$), but this was weaker than that of the

aforementioned indicators. Interestingly, HOMA-IR did not correlate with serum adiponectin (r = -0.303, P = 0.170), which was inversely correlated with waist circumference (r = -0.567, P = 0.007), waist-to-hip ratio (r = -0.700, P < 0.001), and γGT (r = -0.631, P = 0.002), and positively correlated with high-density-lipoprotein-cholesterol (r = 0.473, P = 0.026). On the other hand, there were no significant correlations between HOMA-IR or serum adiponectin with serum AST, ALT, hsCRP, platelet count, hyaluronic acid, APRI, HCV core antigen, ferritin, or transferrin saturation ratio. The HOMA-β was weakly associated with hip circumference (r = 0.452, P = 0.040) and serum TG (r = 0.410, P = 0.042). No similar correlations were found in asymptomatic HBV carriers, demonstrating the existence of a positive and strong association between HOMA-IR/serum adiponectin, waist circumference, and serum γGT and TG in chronically HCV-infected patients.

Discussion

In the current study, there were no significant differences in HOMA-IR and serum adiponectin between non-obese, non-diabetic, non-alcoholic, and non-steatotic HCV and HBV carriers with PNALT. According to multivariate analysis, the presence of HCV was not an independent predictor of IR. HOMA-IR in the HCV carriers was strongly associated with waist circumference and serum γGT and TG, but not with the indicators of obesity (BMI), hepatocyte injury (serum AST or ALT), hepatic fibrosis (platelet count, serum hyaluronic acid, or APRI), iron accumulation (serum ferritin or transferrin saturation ratio), systemic inflammation (serum hsCRP), or amount of HCV core protein. These results support the premise that the presence of HCV alone cannot induce IR. To our knowledge, this is the first study to evaluate HOMA-IR and serum adiponectin in non-obese, non-diabetic HCV carriers with PNALT and compare them with HBV carriers.

We defined the presence of IR as a HOMA-IR of greater than 1.73. Although considerably lower than that in Hispanic and Caucasian populations, this cutoff value is consistent with the results of a previous study using young, lean, healthy individuals (31) that showed a significantly lower insulin sensitivity index in Asian groups compared with other ethnic groups.

Several lines of evidence have shown that the presence of obesity, alcohol consumption, and hepatic steatosis all contribute to the onset of IR (5, 6, 17, 18, 21). To exclude these factors as much as possible, we first selected non-obese hepatitis virus carriers devoid of a history of habitual alcohol intake and hepatic steatosis, and closely matched by BMI and waist-to-hip ratio. Our results revealed that waist circumference, an important anthropometric predictor of visceral fat accumulation (32), was significantly correlated with HOMA-IR and inversely associated with serum adiponectin in HCV carriers. Surprisingly, BMI was not found to strongly affect HOMA-IR in our cohort. Several studies have documented a close relationship between visceral fat accumulation and IR in healthy volunteers (33, 34), and the results of this study support such a strong contribution to IR development in chronically HCV-infected patients as well.

We judged the presence of hepatic steatosis using abdominal US. Although US is a safe, noninvasive, and accurate method of detecting moderate-to-severe steatosis, its diagnostic accuracy declines sharply in cases of mild steatosis (liver fat less than 25%). Indeed, clear differentiation between nonalcoholic steatohepatitis (NASH) with mild steatosis or cryptogenic chronic hepatitis is sometimes difficult by such imaging modalities. We previously reported that in Japanese patients with persistent ALT

elevation, despite no detection of steatosis by US, obesity, hyperferritinemia, and high HOMA-IR are predictors of NASH with mild steatosis (5). The close relationship between IR and hepatic steatosis has also been documented elsewhere in the Asian population (31). In this study, patients with a BMI of more than 25 kg/m^2 were excluded, and most patients demonstrated normal serum ferritin and HOMA-IR. Thus, the possibility that patients with mild steatosis were included is presumably low.

Since advanced fibrosis may also lead to hyperinsulinemia probably due to decreased insulin clearance capacity (11, 12), we also limited our patients to those having no or mild fibrosis, as estimated by platelet count, serum hyaluronic acid, and ultrasonographic findings; it is known that chronic hepatitis C patients presenting with serum hyaluronic acid of less than 50 ng/mL correspond to the absence of severe fibrosis (35). Moreover, more than 90% of HCV carriers with PNALT presenting with platelet count of more than 150 x 10^3/μL with are reported to have normal or mild liver histologies (20). Although percutaneous liver biopsies could not be performed in our patients, selection according to the above strict criteria enabled us to confidently exclude the possibility of IR caused by advanced fibrosis. Low APRIs in both groups also confirm the relevance of our selection criteria.

Under these conditions, we were able to discover that serum γGT was closely associated with HOMA-IR in non-obese, non-diabetic, non-alcoholic, and non-steatotic HCV carriers with PNALT. These results are consistent with those of previous studies in that serum γGT is an important risk indicator for developing metabolic syndrome and type 2 diabetes (36, 37). A positive association between serum γGT and hepatic TNF-α expression has been documented in patients with chronic HCV infection (38). Since an activated TNF-α system is one of the major causes of IR development (15), the close relationship seen between serum γGT and HOMA-IR may partially reflect the local enhancement of TNF-α expression in HCV-infected livers. Although serum γGT is a nonspecific marker of liver injury, it may also become a useful predictor of IR in HCV-infected patients.

Interestingly, serum HCV core protein concentration was not associated with HOMA-IR, which was inconsistent with a previous report demonstrating an association between HOMA-IR and the amount of serum HCV core antigen (14). Although the direct contribution of core protein to the pathogenesis of HCV-induced IR has been shown in transgenic mouse lines (14, 15), recent studies have uncovered that the occurrence of core protein-induced IR is not derived from the protein's intrinsic effect. For example, in mice constitutively expressing HCV core protein, deletion of the proteasome activator PA28γ gene did not induce hyperinsulinemia or IR, despite the

presence of core protein (39). Moreover, we obtained similar results from peroxisome proliferator-activated receptor α-null mice bearing the core protein gene (40). Thus, the results in this study support the notion that the core protein itself does not have the potential to induce IR.

In humans, the relationship between serum adiponectin and the presence of HCV is a matter of controversy. Several studies have reported that low adiponectin is significantly associated with high HOMA-IR in patients with chronic hepatitis C (41, 42). On the other hand, a recent large-scale study has clearly shown that chronic HCV infection has little influence in serum adiponectin (43). We here also demonstrated no correlation between HOMA-IR and serum adiponectin in non-steatotic HCV-infected patients with PNALT, as well as no differences in adiponectin levels compared to matched HBV carriers. Our results lead us to conclude that the probability of HCV itself modulating adiponectin expression is low.

Also in this study, serum hsCRP, a surrogate marker of subclinical systemic inflammation, did not differ between HCV and HBV carriers. It has been documented by a case-control study that serum levels of proinflammatory cytokines inducing IR, such as TNF-α and interleukin-6, were higher in patients with chronic hepatitis C than in those with other causes of hepatitis, despite similar levels of hepatitis activity (19). Thus, activation of TNF-α-mediated pathway may contribute to HCV-specific IR.

The present study suggests the contribution of hepatic inflammatory component to the development of IR in chronic hepatitis C. However, our results do not necessarily mean that IR frequently found in chronic hepatitis C patients is mediated by hepatitis alone. HOMA-IR was reported to be higher in patients with chronic hepatitis C than in those with other causes of hepatitis independently of severity of hepatic inflammation and fibrosis (2, 19), indicating diabetogenic potential of HCV. HCV might lead to latent disturbance of insulin signaling cascade which cannot be detected by a simple indicator (i.e., HOMA-IR), and trigger IR in cooperation with other factors such as hepatitis.

Clinically, it is well-known that increased HOMA-IR is one of the primary predictors of hyporesponsiveness or failure of interferon therapy in persistently HCV-infected patients (44, 45). The demonstration of a strong relationship between HOMA-IR and waist circumference, serum γGT and TG in HCV-infected humans without obesity, diabetes, hepatocyte damage, hepatitis, or obvious steatosis indicates that simple nutritional intervention and exercise to reduce visceral fat mass can further ameliorate the outcome of antiviral therapy. In addition, the combination therapy of insulin-sensitizing TG-lowering agents and interferon injections might prove beneficial. In fact, additional treatment with bezafibrate, a typical TG-lowering agent (46), was

reported to achieve a higher complete response rate with interferon and ribavirin combination therapy (47). Therefore, accurate evaluation of metabolic disturbances, such as visceral fat accumulation and high levels of serum TG and HOMA-IR, and the ensuing steps taken to regulate them, round out a list of therapeutic strategies for HCV-infected patients.

There are some limitations in the present study. First, the sample size is limited. Large-scale case control studies using the same selection criteria will be able to further ascertain the association between HCV infection and development of IR. Second, the patients were selected from a homogenous race (i.e., Japanese), and the pathogenesis of HCV-specific IR might differ between races. Finally, we were not able to access the changes in IR with aging, necessitating further long-term follow-up of our patients to address the issue.

In conclusion, the results of this study demonstrate that the presence of HCV per se cannot induce IR; rather, it may be other factors, such as the presence of active hepatitis, hepatic steatosis or fibrosis, that are important to HCV-specific IR. In addition, waist circumference and serum γGT and TG were strongly associated with HOMA-IR in non-obese, non-alcoholic, and non-steatotic HCV carriers with PNALT, suggesting the likelihood that these parameters are useful and reliable indicators of IR in HCV-infected patients. Although our data offer novel information about the pathogenesis of HCV-specific IR, further large-scale studies are needed to confirm our results.

Acknowledgments

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Figure Legend (Figure on the next page)

Fig. 1. Patient selection criteria

The left and right number in each box shows the number of patients with chronic infection of HBV and HCV, respectively. ALT, alanine aminotransferase; Plt, platelet counts; HA, hyaluronic acid; PNALT, persistently normal serum ALT; BMI, body mass index; FPG, fasting plasma glucose; US, ultrasonography.

Fig. 1

Chronically HBV- or HCV-infected patients $\boxed{161/268}$

$\rightarrow$ ALT $\geq$ 30 U/L or Plt $\leq$ 150 x 10^3/μL
or undergoing treatments $\boxed{104/210}$

$\rightarrow$ HA $\geq$ 50 ng/mL $\boxed{8/4}$

Patients with PNALT $\boxed{49/54}$

(ALT < 30 U/L, Plt > 150 x 10^3/μL, HA < 50 ng/mL for more than 5 years)

$\rightarrow$ BMI $\geq$ 25 kg/m^2 $\boxed{8/2}$
$\rightarrow$ Alcohol consumption $\geq$ 20 g/day $\boxed{1/1}$
$\rightarrow$ FPG $\geq$ 7 μmol/mL or diabetes $\boxed{2/1}$
$\rightarrow$ Steatosis by US $\boxed{8/4}$

Non-obese, non-diabetic, non-alcoholic, and non-steatotic patients with PNALT $\boxed{30/46}$

$\rightarrow$ $\geq$ 65 years in patients with HCV $\boxed{0/14}$
$\rightarrow$ BMI < 15 kg/m^2 in patients with HCV $\boxed{0/2}$

Patients analyzed $\boxed{30/30}$