exhibited SVR rates of almost 100%, patients who became negative within 5–12 weeks exhibited SVR rates of approximately 70%, and patients who became negative within 13–24 weeks exhibited SVR rates of approximately 30%. Therefore, there have been attempts to increase the efficacy of treatment by extending the duration of treatment. Previous reports have shown that the efficacy of treatment can be increased by extending administration to 72 weeks for late viral responder that become RNA negative between weeks 12 and 24 (7–10). From these reports, it is believed that extended administration to late viral responder can assure increases in treatment efficacy. However, considering the physical and financial burdens to the patients, we believe that it would be important to establish the shortest possible treatment duration that exerts maximum efficacy (19). We thus believe that it is necessary to establish more detailed individualized treatment duration rather than extending treatment from 48 to 72 weeks. On the other hand, Drusano *et al.* (15) have reported that in previously untreated genotype 1 patients at least 32–36 weeks of undetectable HCV RNA by quantitative PCR is needed to attain the sustained clearance of HCV. We established the duration of our treatment so that the administration would be performed for 44 weeks after HCV RNA became negative, because the SVR rate of patients who became HCV RNA negative at week 4 was almost 100%. HCV RNA was only measured at weeks 4, 12, and 24 in the previous reports, but in Japan, HCV RNA is often measured every 4 weeks, and treatment is administered while checking the treatment efficacy, so we believe that it is possible to establish shorter periods of durations.

Among the patients in the present study, almost all patients in the standard group that became HCV RNA negative at weeks 4 and 8 exhibited SVR, so we therefore considered 48 weeks of administration to be sufficient. It is reported that high SVR rates have been obtained even with 24 weeks of administration in patients who became RNA negative at week 4 (20,21), 48 weeks may be too long. In the extended group, the patients who became HCV RNA negative at weeks 12 showed response rates of 79%, which were almost the same as those in the standard group (71%). As a comparatively sufficiently high response rate has been obtained in the standard group, our extended therapy may not benefit these patients.

On the other hand, the SVR rate of patients who became negative from weeks 16 to 24 was 9% in the standard group and 78% in the extended group.

Our extended therapy had maximal benefit in these patients. However, as the SVR rate of patients who became HCV RNA negative from weeks 20 and 24 was low (50%), a slightly higher response rate may be obtained through an extended administration of over 68 weeks in such patients, and this will be the subject of future study.

In terms of factors that contribute to SVR, the duration of administration and the time of HCV RNA negative were extracted as the most important factors according to the results of multivariable analysis. As there were no differences in the amount of ribavirin that was administered per day or the amount of peginterferon that was administered per week between the SVR and non-SVR groups, we believe that the duration of administration is important.

We believe that, in the future, it will be important to analyze patients who do not exhibit SVR despite extended administration. In the present study, one of the patients who became HCV RNA negative at week 12 was administered very small dosages because of the occurrence of side effects, but many patients were given full doses of both drugs, and the number of non-SVR patients was small, so the characteristics of the non-SVR patients could not be elucidated (data not shown).

The side effects were the same as those reported in previous reports. Moreover, regarding the time of discontinuation due to side effects, in all patients discontinuation occurred within 48 weeks from the start of administration, and no patients were discontinued during the extended period after 48 weeks. Therefore, we believe that extending administration to 48 weeks or longer would not cause any significant problems in terms of safety.

In conclusion, we believe that our extended administration method is a unique and economical treatment method that is able to achieve a high treatment efficacy.

CONFLICT OF INTEREST

Guarantor of the article: Tatsuya Ide, MD.
Specific author contributions: conception: Tatsuya Ide; study design: Tatsuya Ide, Teruko Arinaga, Michio Sata; participation in patient management and data collection: all authors; contribution to the data acquisition, responsibility for writing the paper, and statistical analysis: Tatsuya Ide. All authors reviewed the paper and approved the final version.
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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Forty-eight week regimen of peginterferon/ribavirin treatment is currently recommended therapy for HCV genotype 1-infected patients. However, there is only a 40–50% likelihood of achieving a sustained virological response (SVR).
- ✓ The efficacy of treatment can be increased by extending administration to 72 weeks for late viral responder that become RNA negative between weeks 13 and 24.
- ✓ It is desirable to tailor the treatment regimen to achieve the SVR more efficiently.

WHAT IS NEW HERE

- ✓ The extended therapy regimen was designed to ensure the patients become HCV RNA negative for 44 weeks.
- ✓ This treatment significantly increased the SVR rate in patients who were HCV RNA negative at 16–24 weeks.
- ✓ This treatment is a unique and economical treatment method.

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CLINICAL STUDIES

Association of exogenous insulin or sulphonylurea treatment with an increased incidence of hepatoma in patients with hepatitis C virus infection

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Keywords
hepatitis C virus – hepatocellular carcinoma – diabetes mellitus – insulin – sulphonylurea

Abbreviations
ALP, alkaline phosphatase; ALT, alanine aminotransferase; APRI, aspartate aminotransferase to platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; γ-GTP, γ-glutamyl transpeptidase; HbA1c, haemoglobin A1c; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HOMA-IR, homeostasis model assessment of insulin resistance; IGF, insulin-like growth factor; LDH, lactate dehydrogenase; OR, odds ratio.

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Abstract

Background: Diabetes mellitus is frequently seen in hepatitis C patients and is often treated with antidiabetic agents that increase serum insulin levels. Because insulin is a growth-promoting hormone, antidiabetic agents could pose a risk for hepatocellular carcinoma (HCC). *Aim:* The aim of this study was to investigate an association between antidiabetic therapies and the incidence of HCC in hepatitis C patients with diabetes mellitus. *Methods:* A nested case–control study was conducted. Participants were recruited from a cohort study, in which patients with hepatitis C were consecutively registered. Participants were assigned to an HCC group ($n = 138$) or a non-HCC group ($n = 103$). To identify independent factors, variables including use of antidiabetic agents were analysed by logistic regression analysis. *Results:* Besides ageing, being male, cirrhosis and hypoalbuminaemia, use of exogenous insulin and a second-generation sulphonylurea were significant independent factors associated with an incidence of HCC [odds ratio (OR) 2.969, 95% confidence interval (CI) 1.293–6.819, $P < 0.0103$ and OR 6.831, 95% CI 1.954–23.881, $P < 0.0026$ respectively). In stratified analyses, the impact of these antidiabetic agents was more evident in patients who were non-cirrhotic than in those who were cirrhotic. *Conclusions:* Exogenous insulin and a second-generation sulphonylurea were independent variables associated with an incidence of HCC in hepatitis C patients with diabetes mellitus. This association was evident in patients who were non-cirrhotic. To verify a causal relationship between these antidiabetic agents and the development of HCC, a prospective cohort study is required.

Type 2 diabetes mellitus is a common metabolic abnormality worldwide and is associated with various complications. For example, cardio-vascular complications are well known for diabetes mellitus (1), while recent epidemiological studies have shown that patients with type 2 diabetes mellitus are highly predisposed to cancer (2). Type 2 diabetes mellitus has been implicated in the development of cancer of the pharynx, oesophagus, colorectum, pancreas, cervix uteri, breast and prostate (2–5). In addition, type 2 diabetes mellitus is also known to be associated with the development of hepatocellular carcinoma (HCC) (6–8).

Type 2 diabetes mellitus is also frequently seen in patients with chronic hepatitis C virus (HCV) infection (9–11). Factors that are involved in the development of type 2 diabetes mellitus include not only life-style choices such as diet and exercise but also hepatic inflammation, fibrosis, steatosis, iron deposition and HCV core protein (10, 12–15). A high prevalence of HCC is seen in patients with HCV infection compared with patients with other

chronic liver diseases. Although the mechanism for HCV-related hepatocarcinogenesis is unclear, an association between type 2 diabetes mellitus and HCV infection could be responsible for the high prevalence of HCC in patients with HCV infection.

Hyperglycaemia is a common feature of type 2 diabetes mellitus. Hyperglycaemia increases oxidative stress, which causes oxidative DNA damage, an initial step in carcinogenesis (16). Moreover, hyperglycaemia is a factor leading to carcinogenesis because of immune suppression through the regulation of T-cell function (17). Thus, hyperglycaemia itself might stimulate hepatocarcinogenesis.

Hyperinsulinaemia combined with insulin resistance is another common feature of type 2 diabetes mellitus. Insulin is a growth-promoting hormone with mitogenic effects (18), and therefore could stimulate hepatocarcinogenesis. An increase in circulating insulin levels is also seen in diabetic patients treated with sulphonylureas or exogenous insulin. Indeed, exogenous insulin injection promotes colonic carcinogenesis in rats (19) and use of exogenous insulin significantly increases the risk of colorectal cancer among diabetic patients (20). Similarly, patients with type 2 diabetes exposed to sulphonylureas and exogenous insulin have a significantly increased risk of cancer-related mortality compared with patients exposed to metformin, a insulin sensitizer (21). Moreover, metformin reduces the risk of cancer in patients with type 2 diabetes (22). These findings suggest that pharmacologic effects of antidiabetic agents on circulating insulin levels play an important role in carcinogenesis. Despite the recognition of this potential link between type 2 diabetes mellitus and hepatocellular carcinoma, a role for antidiabetic agents in hepatocarcinogenesis has not been established.

Accordingly, in this study, we examined a possible association between antidiabetic therapies and an incidence of HCC in patients with HCV infection.

Methods

Study design and participants

A nested case–control study was conducted. Hepatitis C patients with diabetes mellitus were culled from the HCV-related diabetes mellitus study (HDMS), a hospital-based, prospective, multi-centre cohort. Patients with hepatitis C were consecutively recruited from three Japanese hospitals specialized for liver diseases (Kurume University Hospital, Nagata Hospital, and Chikugo City Hospital) from January 2004 to December 2008. Eligible participants were identified from all patients who were aged $\geq$40 years old, and had both a positive result for anti-HCV antibodies and a diagnosis of type 2 diabetes mellitus. The diagnosis of type 2 diabetes mellitus was based on the 2004 American Diabetes Association criteria (23) or use of any anti-diabetic agent. Participants in whom diabetes was diagnosed before age 30 with a positive result for pancreatic beta-cell autoantibodies (antiglutamic acid decarboxylase, anti-insulinoma-associated protein-2 or anti-islet-cell antibodies) were categorized as having type 1 diabetes mellitus and were excluded.

A total of 265 participants provided baseline data. The analysis was performed on the data of 241 participants, after excluding 24 participants because of unavailability of data on glucose metabolism, coincidence with other causes of liver disease such as chronic hepatitis B, autoimmune hepatitis, a metastatic liver tumour, or cholangiocellular carcinoma, taking corticosteroids or a history of pancreatitis or a pancreatic tumour. HCC was diagnosed by ultrasonic-guided biopsy, the non-invasive European Society of Study of the Liver criteria for the diagnosis of HCC or superparamagnetic iron oxide-enhanced magnetic resonance imaging (24, 25). All patients were classified into an HCC or a non-HCC group according to the incidence of HCC.

Informed consent for participation in this study was obtained from each participant. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in prior approval by the Ethics Committee of the Kurume University School of Medicine. None of the participants was institutionalized.

Measurements

Clinical data including age, sex and alcohol intake were collected at the first medical examination. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of the height in metres (kg/m^2).

The diagnosis of cirrhosis was based on a liver biopsy or the aspartate aminotransferase (AST) to platelet ratio index (APRI); serum AST level (U/L)/upper limit of normal AST (33 U/L) $\times$ 100/platelet count ($\times$ 10^4/ml) (26). APRI is one of the models for predicting the stage of liver fibrosis. Patients with APRI values higher than 2.0 were diagnosed as having cirrhosis (26). In stratification analysis, the severity of liver disease was classified into non-cirrhosis or cirrhosis.

Venous blood samples were taken in the morning after a 12-h overnight fast. The following were measured using standard clinical methods: platelet count, plasma glucose, haemoglobin A1c (HbA1c), serum AST, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), albumin and total bilirubin levels (Department of Clinical Laboratory, Kurume University Hospital, Nagata Hospital and Chikugo City Hospital).

Classification of antidiabetic agents

Antidiabetic agents were classified into exogenous insulin (any type of insulin preparation), second-generation sulphonylurea (gliclazide or glybenclamide), third-generation sulphonylurea (glimepiride), α-glucosidase inhibitor (acarbose, voglibose or miglitol), glinide (nateglinide or mitiglinide), biguanide (metformin) or

thiazolidine (pioglitazone) according to each characteristic of these agents.

Statistical analysis

All data are expressed as mean ± standard deviation. Comparisons between the two groups were performed using the Mann–Whitney *U*-test for continuous variables and univariate analysis for discrete variables. In order to evaluate the possible importance of variables, a univariate analysis was performed initially. The relevant variables with univariate *P* values < 0.1 were selected for inclusion in the initial step of logistic analysis. Then, logistic regression analysis was used to identify any independent variable that was related to the incidence of HCC. The variables analysed were age (≥ 60 years old), sex, BMI (≥ 25 kg/m^2), alcohol intake (≥ 50 g/day), the incidence of cirrhosis, use of exogenous insulin, use of a second-generation sulphonylurea, use of biguanide, total bilirubin (≥ 2 mg/dl), albumin (< 3.5 g/dl), AST (≥ 40 U/L), ALT (≥ 40 U/L), LDH (≥ 230 U/L), ALP (≥ 360 U/L), γ-GTP (≥ 50 U/L), platelet count ($< 10 \times 10^4$/μl), fasting plasma glucose (≥ 126 mg/dl) and HbA1c ($\geq 5.9\%$). Stratification analysis was conducted to examine the impact of use of exogenous insulin and a second-generation sulphonylurea on the incidence of HCC. Stratification factors were severity of liver disease (non-cirrhotic patients, cirrhotic patients), hypoalbuminaemia (≥ 3.5 g/dl or < 3.5 g/dl) and sex (male or female). All the statistical tests were two-sided, and a *P* value of < 0.05 was considered to be statistically significant.

Results

Comparison of the characteristics and use of antidiabetic agents between hepatocellular carcinoma and non-hepatocellular carcinoma groups in hepatitis C patients with diabetes mellitus

Age, prevalence of males, the incidence of cirrhosis and fasting plasma glucose were significantly higher in the HCC group compared with those in the non-HCC group (Table 1).

There were no significant differences in the HbA1c levels between two groups. However, use of anti-diabetic agents was more frequent in the HCC group than that in the non-HCC group ($P = 0.0030$; Table 1). In order to investigate an association between HCC and the pharmacologic effects of anti-diabetic agents, anti-diabetic agents were classified into seven subgroups according to the characteristics of each agent: exogenous insulin, second-generation sulphonylurea, third-generation sulphonylurea, α-glucosidase inhibitor, glinide, biguanide or thiazolidine. There were no significant differences in the use of a third-generation sulphonylurea, α-glucosidase inhibitor, glinide, biguanide or thiazolidine between HCC and non-HCC groups. However, use of exogenous insulin and a second-generation sulphonylurea were significantly more frequent in the HCC group than those in the non-HCC group ($P = 0.0020$ and $P = 0.0003$, respectively; Table 1).

Table 1. Comparison of characteristics and use of anti-diabetic agents between hepatocellular carcinoma and non-hepatocellular carcinoma groups in hepatitis C patients with diabetes mellitus

	HCC ($n = 138$)	Non-HCC ($n = 103$)	*P* value
Age (year)	68.8 ± 8.0	64.7 ± 10.3	0.0032
Male	103	60	0.0083
BMI (kg/m^2)	22.9 ± 3.2	22.8 ± 3.5	0.4595
Excess alcohol intake (≥ 50 g/day)	29	12	0.0590
Cirrhosis	101	34	< 0.0001
Total bilirubin (mg/dl)	1.37 ± 1.96	1.09 ± 1.46	0.1171
Albumin (g/dl)	3.39 ± 0.53	3.82 ± 0.56	< 0.0001
AST (U/L)	65.4 ± 33.0	55.4 ± 32.8	0.0027
ALT (U/L)	56.9 ± 32.6	56.4 ± 40.5	0.3534
LDH (U/L)	229.6 ± 70.1	214.7 ± 65.9	0.0286
ALP (U/L)	392.0 ± 213.3	343.0 ± 161.3	0.0547
γ-GTP (U/L)	130.6 ± 239.5	74.8 ± 80.4	0.0046
Platelet count (× 10^4/μl)	10.5 ± 5.2	12.9 ± 5.2	0.0002
Fasting plasma glucose (mg/dl)	163.4 ± 70.1	146.1 ± 60.5	0.0230
HbA1c (%)	6.6 ± 1.1	7.1 ± 1.7	0.0905
Use of antidiabetic agents	98	53	0.0030
Exogenous insulin	43	14	0.0020
Second-generation sulphonylurea	26	4	0.0003
Third-generation sulphonylurea	22	20	0.4969
α-glucosidase inhibitor	25	15	0.4898
Glinide	8	11	0.2266
Biguanide	4	5	0.5024
Thiazolidine	1	4	0.1670

All data are expressed as mean ± standard deviation.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GTP, glutamyl transpeptidase; HCC, hepatocellular carcinoma; LDH, lactate dehydrogenase.

Variables associated with the incidence of hepatocellular carcinoma in hepatitis C patients with diabetes mellitus

In the univariate analysis, age (≥ 60 years old), being male, cirrhosis, albumin (< 3.5 g/dl), AST (≥ 40 U/L), LDH (≥ 230 U/L), ALP (≥ 360 U/L), platelet count ($< 10 \times 10^4$/μl) and HbA1c ($\geq 5.9\%$) were significant variables associated with the incidence of HCC (Table 2). In addition, use of exogenous insulin and a second-generation sulphonylurea were significant variables associated with the incidence of HCC [odds ratio (OR) 2.877, 95% confidence interval (CI) 1.474–5.617, $P < 0.0020$ and OR 5.746, 95% CI 1.938–17.038 respectively: Table 2).

In the logistic regression analysis, age (≥ 60 years old), being male, cirrhosis and albumin (< 3.5 g/dl) were independent factors associated with a greater incidence of HCC (Table 2). Moreover, use of exogenous insulin

Table 2. Univariate and logistic regression analyses for the incidence of hepatocellular carcinoma in all patients ($n = 241$)

	Univariate analysis			Logistic regression analysis		
Variable	HR	95% CI	*P* value	HR	95% CI	*P* value
Age (≥60 years)	2.270	1.221–4.222	0.0096	2.781	1.231–6.283	0.0139
Male	2.109	1.219–3.649	0.0076	3.075	1.511–6.258	0.0019
BMI (≥25 kg/m^2)	1.085	0.559–2.105	0.8092			
Excess alcohol intake (≥50 g/day)	2.018	0.974–4.179	0.0588			
Cirrhosis	5.540	3.173–9.672	< 0.0001	3.366	1.465–7.731	0.0042
Total bilirubin (≥2 mg/dl)	2.537	0.801–8.028	0.1133			
Albumin (< 3.5 g/dl)	5.078	2.843–9.070	< 0.0001	3.008	1.373–6.591	0.0059
AST (≥40 U/L)	2.447	1.403–4.267	0.0016	1.690	0.797–3.582	0.1709
ALT (≥40 U/L)	1.441	0.856–2.427	0.1694			
LDH (≥230 U/L)	1.919	1.099–3.350	0.0220	0.936	0.436–2.008	0.8650
ALP (≥360 U/L)	1.912	1.100–3.325	0.0216	1.044	0.499–2.187	0.9084
γ-GTP (≥50 U/L)	1.565	0.929–2.637	0.0926			
Platelet count (< 10×10^4/μl)	2.000	1.177–3.398	0.0104	0.913	0.401–2.079	0.8285
Fasting plasma glucose (≥126 mg/dl)	1.559	0.9296–2.624	0.0945			
HbA1c (≥5.9%)	0.451	0.219–0.929	0.0308	0.654	0.268–1.596	0.3511
Use of exogenous insulin	2.877	1.474–5.617	0.0020	2.969	1.293–6.819	0.0103
Use of second-generation sulphonylurea	5.746	1.938–17.037	0.0016	6.831	1.954–23.881	0.0026
Use of third-generation sulphonylurea	0.787	0.404–1.535	0.4823			
Use of α-glucosidase inhibitor	1.298	0.646–2.609	0.4641			
Use of glinide	0.515	0.199–1.330	0.1702			
Use of biguanide	0.585	0.153–2.235	0.4332			
Use of thiazolidine	0.181	0.020–1.641	0.1285			

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; GTP, glutamyl transpeptidase; HR, hazard ratio; LDH, lactate dehydrogenase.

and a second-generation sulphonylurea were also identified as independent variables associated with an incidence of HCC (OR 2.969, 95% CI 1.293–6.819, $P = 0.0103$, OR 6.831, 95% CI 1.954–23.881, $P = 0.0026$ respectively; Table 2). Use of a second-generation sulphonylurea showed the highest OR among all the variables (Table 2).

Variables associated with the incidence of hepatocellular carcinoma in stratification analysis by severity of liver disease

All patients were stratified into two subgroups: a non-cirrhosis or a cirrhosis group. In patients with cirrhosis, age (≥60 years old) and albumin (< 3.5 g/dl) were identified as independent factors associated with the incidence of HCC in the logistic regression analysis (Table 3). However, use of any antidiabetic agent was not an independent factor associated with the incidence of HCC in patients with cirrhosis (Table 3).

In non-cirrhotic patients, not only being male but also use of exogenous insulin and a second-generation sulphonylurea were determined to be independent variables associated with a greater incidence of HCC (Table 3).

Variables associated with the incidence of hepatocellular carcinoma in stratification analysis by hypoalbuminaemia

All patients were stratified into two subgroups: ≥3.5 g/dl of albumin or < 3.5 g/dl of albumin group. In patients with < 3.5 g/dl of the albumin, being male and age (≥60 years old) were associated with the incidence of HCC in the logistic regression analysis (Table 4). Use of exogenous insulin or a second-generation sulphonylurea was not determined to be an independent variable, while use of biguanide was negatively associated with the incidence of HCC (Table 4).

In patients with ≥3.5 g/dl of albumin, not only being male and cirrhosis but also use of a second-generation sulphonylurea were determined to be independent variables associated with a greater incidence of HCC (Table 4). Use of a second-generation sulphonylurea showed the

Table 3. Logistic regression analysis for the incidence of hepatocellular carcinoma by stratification according to severity of liver disease

	Logistic regression analysis		
Variables	OR	95% CI	*P* value
Chronic hepatitis ($n = 107$)			
Male	6.150	1.705–22.185	0.0055
Use of exogenous insulin	4.142	1.072–16.007	0.0393
Use of second-generation sulphonylurea	4.822	0.963–24.144	0.0556
AST (≥40 U/L)	1.506	0.530–4.285	0.4423
γ-GTP (≥50 U/L)	2.234	0.759–6.578	0.1444
Albumin (< 3.5 g/dl)	3.632	0.940–14.031	0.0614
Cirrhosis ($n = 134$)			
Age (≥60 years)	3.357	1.335–8.440	0.0100
Albumin (< 3.5 g/dl)	2.402	1.061–5.436	0.0355

AST, aspartate aminotransferase; CI, confidence interval; GTP, glutamyl transpeptidase, OR, odds ratio.

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Table 4. Logistic regression analysis for the incidence of hepatocellular carcinoma by stratification according to hypoalbuminaemia

	Logistic regression analysis		
Variable	OR	95% CI	*P* value
≥3.5 g/dl of albumin (*n* = 139)			
Male	2.536	1.066–6.034	0.0353
Cirrhosis	2.830	1.096–7.308	0.0317
Use of exogenous insulin	2.557	0.973–6.718	0.0567
Use of second-generation sulphonylurea	5.195	1.338–20.171	0.0173
AST (≥40 U/L)	1.602	0.696–3.691	0.2682
LDH (≥230 U/L)	1.777	0.696–4.535	0.2289
Platelet count (< 10×10^4/μl)	1.200	0.445–3.237	0.7183
< 3.5 g/dl of albumin (*n* = 102)			
Male	4.922	1.562–15.502	0.0065
Age (≥60 years)	3.357	2.178–28.454	0.0016
Use of biguanide	0.060	0.004–0.846	0.0371

AST, aspartate aminotransferase; CI, confidence interval; LDH, lactate dehydrogenase; OR, odds ratio.

highest OR in patients with ≥3.5 g/dl of albumin (OR 5.195, 95% CI 1.338–20.171, $P = 0.0173$; Table 4).

Variables associated with the incidence of hepatocellular carcinoma in stratification analysis by sex

All patients were stratified into two subgroups: a male or a female group. In male patients, age (≥60 years old) and albumin (< 3.5 g/dl) were identified as independent factors associated with the incidence of HCC in the logistic regression analysis (Table 5). Moreover, use of a second-generation sulphonylurea was also identified as an independent variable associated with a greater incidence of HCC (OR 4.267, 95% CI 1.046–17.412, $P = 0.0431$; Table 5).

In female patients, cirrhosis was identified as an independent factor associated with an incidence of HCC in the logistic regression analysis (Table 5). Use of a second-generation sulphonylurea was also identified as an independent variable associated with a higher incidence of HCC in female patients and its OR was higher than that in male patients (Table 5).

Discussion

We conducted a hospital-based nested case–control analysis in order to identify variables associated with an increasing incidence of HCC. Besides known variables such as ageing, being male, cirrhosis, and hypoalbuminaemia, we found that use of exogenous insulin and a second-generation sulphonylurea that increase circulating insulin levels were independent variables associated with a greater incidence of HCC. In addition, the impact of the use of exogenous insulin and a second-generation sulphonylurea was more evident in patients who did not have cirrhosis or showed ≥3.5 g/dl of albumin.

Table 5. Logistic regression analysis for the incidence of hepatocellular carcinoma by stratification according to sex

	Logistic regression analysis		
Variable	OR	95% CI	*P* value
Male (*n* = 163)			
Age (≥60 years)	2.807	1.114–7.073	0.0286
Cirrhosis	2.298	0.805–6.564	0.1201
Use of exogenous insulin	2.195	0.827–5.825	0.1143
Use of second-generation sulphonylurea	4.267	1.046–17.412	0.0431
AST (≥40 U/L)	1.665	0.678–4.087	0.2656
LDH (≥230 U/L)	1.094	0.434–2.756	0.8486
ALP (≥360 U/L)	1.482	0.605–3.630	0.3890
Platelet count (< 10×10^4/μl)	0.717	0.249–2.067	0.5383
Albumin (< 3.5 g/dl)	3.516	1.299–9.518	0.0133
Total bilirubin (≥2 mg/dl)	0.986	0.095–10.199	0.9905
Female (*n* = 78)			
Cirrhosis	16.710	2.743–101.785	0.0023
Use of exogenous insulin	4.985	0.868–28.618	0.0716
Use of second-generation sulphonylurea	50.993	3.011–863.633	0.0065
HbA1c (≥5.9%)	0.472	0.083–2.682	0.3968
LDH (≥230 U/L)	0.753	0.169–3.366	0.7107
Platelet count (< 10×10^4/μl)	1.800	0.393–8.247	0.4491
Albumin (< 3.5 g/dl)	1.740	0.348–8.700	0.4998

ALP, alkaline phosphatase; AST, aspartate aminotransferase; CI, confidence interval; GTP, glutamyl transpeptidase; LDH, lactate dehydrogenase; OR, odds ratio.

Use of antidiabetic agents was a variable associated with a greater incidence of HCC in this study. Thus, we found a possible association between antidiabetic agents and the risk of HCC. Among antidiabetic agents, use of exogenous insulin and a second-generation sulphonylurea were significant variables associated with the incidence of HCC. Hyperinsulinaemia combined with insulin resistance is considered as a promoter for hepatocarcinogenesis and tumour growth (27, 28). Because exogenous insulin and a second-generation sulphonylurea increase circulating insulin levels, we hypothesize that the use of these antidiabetic agents accelerates the development of HCC in patients with HCV infection. Use of a third-generation sulphonylurea or an α-glucosidase inhibitor was not a variable associated with the incidence of HCC in this study. A third-generation sulphonylurea is categorized as a sulphonylurea because it contains a sulphonylurea structure. However, a third-generation sulphonylurea is known to improve hyperinsulinaemia through extra-pancreatic effects (29). In fact, a preliminary study in our HDMS cohort showed that treatment of the third generation sulphonylurea causes a reduction of serum insulin levels in hepatitis C patients with diabetes mellitus (data not shown). α-glucosidase inhibitors reduce post-prandial glucose elevation by delaying the release of glucose from disaccharides and complexes of carbohydrates and do not promote insulin secretion

(30). In addition, use of biganide, an insulin sensitizer, was negatively associated with the incidence of HCC in hepatitis C patients with < 3.5 g/dl of albumin. Furthermore, subanalysis of our database showed that serum fasting insulin levels and the homeostasis model assessment of insulin resistance (HOMA-IR) value, an index for insulin resistance, were significantly higher in the HCC group compared with that in the non-HCC group [fasting insulin levels; HCC group 16.1 ± 16.8 μIU/ml ($n = 90$); non-HCC group 12.3 ± 11.9 μIU/ml ($n = 88$); $P = 0.0036$, HOMA-IR value; HCC group 4.72 ± 4.95; non-HCC group 3.94 ± 4.35; $P = 0.0404$; data not shown], although serum insulin levels were not routinely measured in all patients. In good agreement with our findings, the significance of hyperinsulinaemia or the use of exogenous insulin has been associated with the development of colon cancer (20, 31). Thus, the characteristics of antidiabetic agents, subanalysis for serum fasting insulin levels and previously reported facts support our hypothesis.

Recently, in patients with type 2 diabetes mellitus, a direct association of HCC with insulin and sulphonylurea treatment has been reported by Donadon *et al.* (32, 33). Our results were in good agreement with the previous report and further provided new information for the impact of the use of exogenous insulin and a second-generation sulphonylurea. By focusing on hepatitis C patients with diabetes mellitus, a more homogenous group, we found that the use of exogenous insulin and a second-generation sulphonylurea were more strongly associated with an incidence of HCC in patients who were not cirrhotic or showed ≥ 3.5 g/dl of albumin than in patients who were cirrhotic or showed < 3.5 g/dl of albumin. Severity of liver disease is also one of the strongest factors of hepatocarcinogenesis. Therefore, in cirrhotic patients, carcinogenic activity of exogenous insulin and a second-generation sulphonylurea may be drowned out by the carcinogenic activities of cirrhosis. Similar findings were obtained in patients with hypoalbuminaemia (< 3.5 g/dl of albumin), a parameter for severity of liver disease. On the other hand, risk for developing HCC is decreased in patients with non-cirrhotic liver disease (34, 35) and ≥ 3.5 g/dl of albumin (36). Thus, we assume that hepatocarcinogenic activities of exogenous insulin and a second-generation sulphonylurea stand out in patients who have such negative factors for the development of HCC. Although sex affects the development of HCC and females are less prone to HCC than males (34, 37), the numbers of the female subset were small and the confidence intervals were large in this study. Therefore, we have to be cautious when interpreting the data and further elucidation is required for sex differences in an association between antidiabetic agents and an increased incidence of HCC.

Kath *et al.* (38) examined an association between the incidence of malignancy and the use of exogenous insulin and reported that insulin treatment is not a risk factor for developing malignancy. Although the reason for this discrepancy is unclear, it might be explained by the fact that only 28 patients developed cancers from 2720 patients in their study. A relatively small number of patients with cancers is one possible reason. More recently, Colhoun and the Scottish Diabetes Research Network (SDRN) Epidemiology Group reported that exogenous insulin use is not associated with an increased risk of site-specific cancers (39). The reason for this discrepancy is also unclear. However, a possible explanation is that the surveyed cancers are different from HCC. In their study, breast, prostate, colorectal, lung and pancreatic cancers were examined. Accordingly, use of exogenous insulin and a second-generation sulphonylurea may only have a significant role in the development of cancer when patients have carcinogenic factors such as an HCV infection. In support of this hypothesis, Donadon *et al.* (32, 33) recently reported a direct association of HCC with insulin and sulfonylurea treatment.

Mechanisms underlying the association of use of exogenous insulin and a second-generation sulphonylurea with HCC risk are uncertain. However, several possibilities exist. Firstly, insulin is an important mitogen and stimulates cell proliferation (18). Insulin directly upregulates intracellular molecules involved in cell proliferation such as mitogen-activated protein kinase by binding to insulin receptors (40). In addition, suppressors of intracellular insulin signalling such as tensin homology deleted on chromosome 10 (41) and SH2 domain-containing inositol phosphatase-2 (7) are downregulated in HCC and therefore insulin effects are considered to be potentiated in HCC. Secondly, insulin also binds insulin-like growth factor (IGF)-1 receptor (42), resulting in the activation of tyrosine kinase and a cascade of intracellular responses. Moreover, insulin inhibits the binding of IGF-1 to IGF-binding proteins and the subsequent increase in IGF-1 levels. The IGF system is a potent growth regulator closely associated with carcinogenesis (43).

Use of a second-generation sulphonylurea was more associated with the incidence of HCC than use of exogenous insulin in all analyses of this study. The reason for the difference in the incidence of HCC between these antidiabetic agents is uncertain. However, one would think that the route of insulin delivery (portal vein or subcutaneous tissue) is a possible reason, because insulin actions in the liver depend on the insulin concentration in the portal vein rather than that in the peripheral vein. Alternatively, sulphonylurea increases not only endogenous insulin secretion but also its precursors which might have mitogenic effects by themselves (44). Similarly, Donadon *et al.* (33) reported that the prevalence of sulphonylurea treatment is higher than that of insulin treatment in patients with HCC.

The main limitation of this study is the study design. A nested case–control analysis is not ideally suited to examine the causal relationship between anti-diabetic agents and the development of HCC. However, the incidence of HCC has rapidly increased over the past 20 years, making HCC one of the fastest-growing causes of

cancer-related death (34). Thus, possible factors associated with the incidence of HCC should be urgently determined. A nested case–control analysis has the advantages of prompt elucidation for association between antidiabetic agents and the development of HCC, and we used a nested case–control analysis in this study. However, some confounding factors including a selection bias may exist in this study and prospective long-term cohort studies are needed. Another limitation of this study is that we did not clarify the types of exogenous insulin. The mitogenic potency of insulin glargine (21^{A}-Gly-30^{B}a-L-Arg-30^{B}b-L-Arg-human insulin) is eight-fold higher than that of human insulin (45). Insulin glargine has recently been reported to increase the incidence rate of cancer (39, 46, 47). However, the carcinogenic activity of glargine is still controversial (48–50) and further study should be focused on an association between types of insulin analogues and the incidence of HCC.

In conclusion, we found that the use of exogenous insulin and a second-generation sulphonylurea was an independent variable associated with an incidence of HCC using a hospital-based nested case–control analysis. In addition, an association between the use of these antidiabetic agents and the incidence of HCC was more evident in patients who were non-cirrhotic or showed ≥ 3.5 g/dl of albumin.

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Branched-Chain Amino Acids and Pigment Epithelium-Derived Factor: Novel Therapeutic Agents for Hepatitis C Virus-Associated Insulin Resistance

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Abstract: Recent clinical studies have shown that patients with chronic liver disease are insulin resistant. Of all etiologies of chronic liver disease including non-alcoholic fatty liver disease, the one that causes the most sever insulin resistance is hepatitis C virus (HCV) infection. Since insulin resistance promotes inflammatory and fibrogenic reactions in the liver, thus leading to the development of liver cirrhosis and hepatocellular carcinoma (HCC) in patients with HCV infection, amelioration of insulin sensitivity may inhibit the progression of HCV-associated liver disease, and could improve the survival of these patients. HCV directly causes insulin resistance through HCV core protein-elicited proteasomal degradation of insulin receptor substrates and subsequent inactivation of intracellular insulin signaling molecules such as Akt. Furthermore, tumor necrosis factor-alpha (TNF-α) and/or triglyceride accumulation-induced nuclear factor-κB (NF-κB) activation in the liver is shown to play a role in insulin resistance in patients with HCV-related chronic liver disease as well. We, along with others, have recently found that branched-chain amino acids (BCAAs) and pigment epithelium-derived factor (PEDF) could improve the HCV-associated insulin resistance *via* suppression of NF-κB and preservation of insulin signaling pathway. In this review, we discuss the mechanisms for the actions of BCAAs and PEDF, and their clinical implications in insulin resistance of chronic liver disease in patients with HCV infection. We also discuss here which chemical structures could contribute to insulin-sensitization in patients with HCV infection.

Keywords: Hepatitis C virus, insulin resistance, branched-chain amino acids, pigment epithelium-derived factor, insulin receptor substrate, suppressor of cytokine signaling, nuclear factor-kappaB, peroxisome proliferator-activated receptor.

INTRODUCTION

Worldwide, more than 170 million people are infected with hepatitis C virus (HCV) [1-3]. HCV infection causes chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC), and HCV-associated liver disease is currently one of the most common reasons for receiving liver transplantation [4]. Therapeutic options, including a combination therapy with pegylated interferon and ribavirin, are far from satisfactory. They lead to a successful outcome in only about 50% of patients with chronic HCV infection because of severe adverse effects [5, 6]. In addition, patients with HCV infection are not always candidates for interferon-based therapies [7]. Thus, HCV is still a main cause of death in chronic liver disease patients [8, 9] and, therefore, the development of new therapeutic approaches is urgently desired.

Recent clinical studies have shown that patients with chronic liver disease are insulin resistant. Of all etiologies of chronic liver disease including non-alcoholic fatty liver disease, the one that causes the most sever insulin resistance is HCV infection [10-17]. Insulin resistance decreases response to antiviral treatment [18-21], promotes progression of hepatic fibrosis [22-24] and esophageal varices [25], increases the risk for the development of HCC [26-29], and is also a sign for poor prognosis in patients with HCV infection [30]. Furthermore, insulin resistance is associated with extrahepatic manifestations of HCV infection such as lichen planus, abnormal thyroid function, and rheumatoid arthritis as well [31, 32]. These observations suggest that insulin resistance plays a pathological role in various intrahepatic and extrahepatic derangements in patients with HCV infection and is a novel therapeutic target for improving the survival and quality of life in these patients.

Although awareness of the health risk of insulin resistance and the development of diabetes has increased, no common pharmaceutical agents are yet available for treating HCV-associated insulin resistance. Exogenous insulin injection and oral hypoglycemic agents such as sulfonylureas are not suitable remedies for the treatment of HCV-associated insulin resistance because (1) most of the patients with HCV infection are hyperinsulinemic, rather than hypoinsulinemic [10, 15-21, 24, 25, 31-37], and (2) insulin is a growth–promoting hormone [38, 39] and use of exogenous insulin or sulfonylureas may be associated with an increased incidence of HCC [35, 40-43]. Although biguanides and thiazolidinediones could improve insulin resistance, and a recent clinical trial shows that metformin improves rapid viral response rate of patients with HCV infection treated by combination therapy with peginterferon and ribavirin [44], they are not always recommended for patients with HCV infection. Indeed, biguanides could predispose to lactic acidosis in patients with severe liver dysfunction [45]. Thiazolidinedione may cause overproduction of hydrogen peroxide and subsequent severe hepatotoxicity in some patients [46]. Lactic acidosis and severe hepatotoxicity are rare adverse effects, however, they sometimes become life-threatening complications in patients with chronic liver diseases.

HCV directly causes insulin resistance through HCV core protein-elicited proteasomal degradation of insulin receptor substrates and subsequent inactivation of intracellular insulin

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signaling molecules such as Akt. Furthermore, activation of tumor necrosis factor-alpha (TNF-α) and/or triglyceride accumulation-induced nuclear factor-κB (NF-κB) in the liver is shown to play a role in the insulin resistance of patients with HCV-related chronic liver disease as well. We, along with others, have recently found that branched-chain amino acids (BCAAs) and pigment epithelium-derived factor (PEDF) could improve HCV-associated insulin resistance *via* suppression of NF-κB and preservation of insulin signaling pathways. In this review, we discuss the mechanisms underlying the actions of BCAAs and PEDF, and their clinical implications in insulin resistance of patients with HCV infection. We also discuss here which chemical structures could contribute to insulin-sensitization in patients with HCV infection.

MOLECULAR PATHWAY OF INSULIN SIGNALING

Insulin is one of the anabolic hormones which regulate not only glucose metabolism, but also protein synthesis, lipid metabolism and cell proliferation through activation of various intracellular signaling molecules as shown in Fig. (**1**) [47-50]. Insulin binds to the extracellular α subunit of the insulin receptor and subsequently causes conformational changes of the insulin receptor, thus leading to the activation of tyrosine kinase domain in the β subunit of the insulin receptor [51]. Activation of the tyrosine kinase of the insulin receptor leads to a phosphorylation of tyrosine residues in the insulin receptor substrate (IRS), a central molecule of intracellular insulin signaling, and then to two major signaling pathways; the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) signaling pathway and mitogen-activated protein kinase (MAPK) signaling pathway [51].

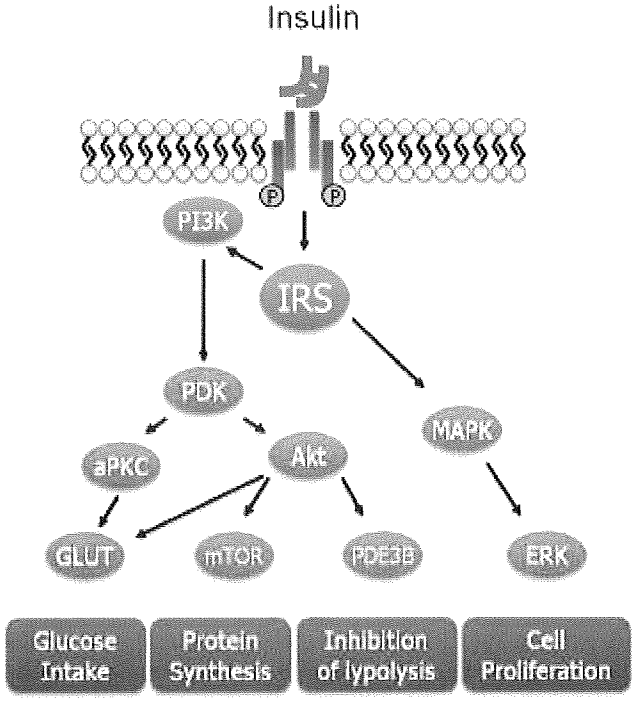

Fig. (1). Molecular pathways of insulin signaling. Abbreviations; aPKC, atypical protein kinase C; ERK, extracellular signal-regulated kinase; GLUT, glucose transporter; IRS, insulin receptor substrates; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; PDE3B, phosphodiesterase 3B; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase.

The PI3K cascade transmits insulin signaling. PI3K activates phosphoinositide-dependent kinase and initiates the activation of atypical protein kinase C (aPKC), resulting in glucose uptake through glucose transporter (GLUT) [52] and glycogen synthesis [53]. Phosphoinositide-dependent kinase (PDK) also activates Akt, leading to activation of its downstream effectors, GLUT 4, mammalian target of rapamycin (mTOR), and phosphodiesterase 3B (PED3B) and to subsequent glucose uptake, protein synthesis and inhibition of lipolysis, respectively [51, 54-56]. The MAPK kinase cascade is involved in cell proliferation through activation of extracellular signal-regulated kinase (ERK) [57].

MOLECULAR MECHANISMS OF HCV-ASSOCIATED INSULIN RESISTANCE

Insulin resistance is defined as an insensitivity of cells to the effects of insulin [54]. Under insulin resistance conditions, post-receptor insulin signaling cascades are disturbed [58]. Obesity is a well-known causative factor for insulin resistance, and inflamed adipose tissues release fatty acids and cytokines resulting in the impairment of intracellular insulin signals [59]. In addition, recent studies have shown that HCV itself could directly elicit insulin resistance [10, 16, 17, 20, 21, 23, 36, 60].

Down-Regulation of IRS1 and IRS2 through Up-Regulation of Suppressor of Cytokine Signaling (SOCS) Proteins

We have previously found that HCV core protein induces nuclear translocation of signal transducer and activation of transcription (STAT) 3 and subsequent up-regulation of SOCS proteins in various hepatoma cell lines [16]. The SOCS family of proteins has functional similarities and similar structural characteristics including a "SOCS box", a unique NH2-terminal domain of variable length, a central Src homology 2 domain, and a COOH-terminal [61-63]. The SOCS box acts as an adaptor to facilitate the ubiquitination of signaling proteins and their subsequent targeting to the proteasome by complexing with Elongins B and C [64, 65]. Since IRS1 and IRS2 are down-regulated in livers from HCV-core transgenic mice and in livers of patients with HCV infection [16, 20], HCV core-induced SOCS3 up-regulation may promote proteasomal degradation of IRS1 and IRS2 through ubiquitination, thus causing insulin resistance in patients with HCV infection as shown in Fig. (**2A**). The following observations further implicate of SOCS3 in HCV-elicited insulin resistance; 1) carbobenzoxy- L-leucyl-L-leucyl-L-leucinal, a potent proteasomal proteolysis inhibitor, inhibits HCV core-induced reductions in IRS1 and IRS2 of HepG2 cells [16], 2) ubiquitination of IRS1 and IRS2 is increased by transfection of HCV core [16], 3) HCV core does not cause down-regulation of IRS1 and IRS2 in SOCS3$^{-/-}$ mouse embryonic fibroblast cells [16], and 4) SOCS-3 immunoreactivity in HCV-infected liver was significantly increased in non-responders to interferon therapy compared with responders [66, 67].

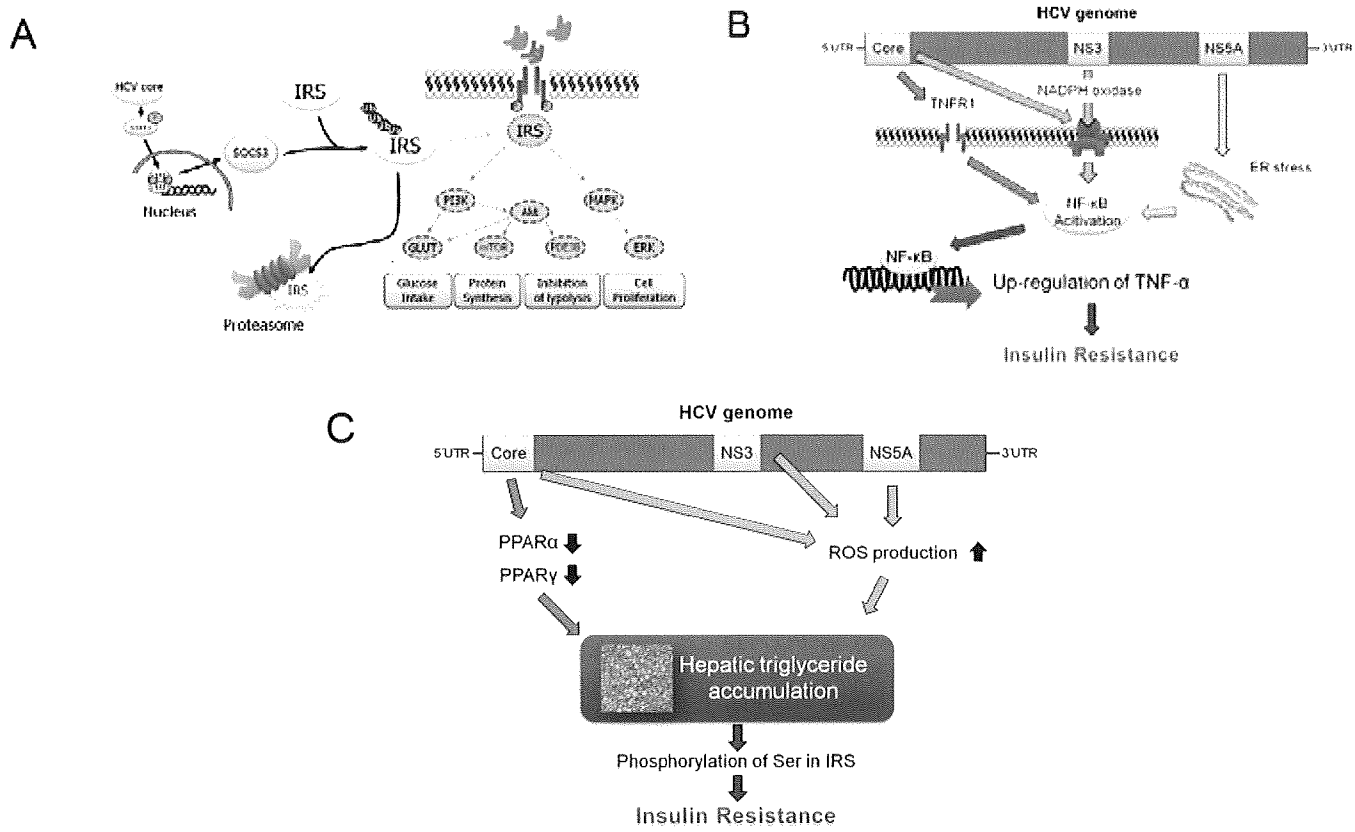

Fig. (2). Molecular mechanisms of HCV-associated insulin resistance. (**A**) Down-regulation of IRS1 and IRS2 through up-regulation of SOCSs. (**B**) Involvement of hepatic activation of NF-κB. (**C**) Participation of triglyceride accumulation in the liver. Abbreviations; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GLUT, glucose transporter; HCV, hepatitis C virus; IRS, insulin receptor substrates; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappaB; NS, nonstructural protein; PDE3B, phosphodiesterase 3B; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF-α receptor 1; TNFR1, TNF-α receptor 1; Ub, ubiquitin, UTR; untranslated region.

Increased TNF-α Expression

Increased TNF-α expression is also involved in HCV-associated insulin resistance [68-71]. HCV core protein binds to the TNF-α receptor 1 (TNFR1), thus activating hepatic NF-κB as shown in Fig. (**2B**) [72, 73]. In addition, HCV core protein and HCV nonstructural protein (NS) 3 stimulate reactive oxygen species (ROS) production through activation of NADPH oxidase in HCV-infected cells [74-78]. ROS, in turn, activates NF-κB and subsequently up-regulates TNF-α expression [79-83], which could cause serine phosphorylation of IRSs and decrease the expression of the glucose transporter (GLUT) in peripheral tissues, thus leading to the development of insulin resistance as shown in Fig. (**2B**) [71]. HCV NS5A induces endoplasmic reticulum stress and also elicits NF-κB activation through Zeta-chain-associated protein kinase 70-mediated tyrosine phosphorylation of inhibitor of NF-κB-α [84].

Hepatic Triglyceride Accumulation

HCV core protein leads to hepatic triglyceride accumulation in transgenic mice, thus suggesting the direct steatogenic effect of HCV [85, 86]. Hepatic triglyceride accumulation is associated with resistance to antiviral treatments in patients with HCV chronic liver disease [87]. Furthermore, increased hepatic triglyceride activates various stress kinases, thus leading to phosphorylation of serine residues in the IRS1 and IRS2 proteins and subsequently causing insulin resistance [88]. Although the mechanism for HCV-associated triglyceride accumulation is not fully elucidated, HCV core protein and structural proteins cause hepatic triglyceride accumulation through increased ROS production as shown in Fig. (**2C**) [77, 89].

Peroxisome proliferator-activated receptors (PPARs) play a crucial role in lipid metabolism in the liver [88, 90-94]. Yamaguchi *et al.* found that HCV core decreased PPARα expression and down-regulated various lipid metabolism-associated gene expressions, including multidrug resistance protein 2, carnitine palmitoyl transferase, and acyl-CoA oxidase, thus leading to hepatic triglyceride accumulation as shown in Fig. (**2C**) [94]. PPARγ is also a key regulator of hepatic lipid metabolism [92]. Activation of PPARγ increases mRNA levels of representative genes of fatty acid oxidation such as acyl-CoA oxidation and reduces triglyceride accumulation in the liver [90]. Liver-specific disruption of PPARγ reduces hepatic triglyceride content in leptin-deficient A-ZIP/F-1 mice, an animal model of type 2 diabetes mellitus, and in wild type mice [93, 95]. De Gottardi *et al.* reported that PPARγ mRNA was decreased in the liver of

patients with HCV infection and was associated with the severity of hepatic steatosis [91]. Furthermore, HCV core protein reduces expression levels of PPARγ and increased triglyceride accumulation in a hepatoma cell line [91].

Thus, amelioration of insulin signaling by down-regulation of SOCS, suppression of TNF-α expression, and hepatic triglyceride accumulation is a novel therapeutic target for HCV-associated insulin resistance.

BCAAs

BCAAs include three amino acids with aliphatic side-chains and comprise the three essential amino acids that cannot be synthesized endogenously in humans; valine, leucine, and isoleucine, as shown in Fig. (**3**). BCAAs are constituents of protein like other amino acids and are required for protein synthesis. In addition, BCAAs are known to have some relevant pharmacologic properties in muscle-protein synthesis, immune system functioning, and ammonia metabolism.

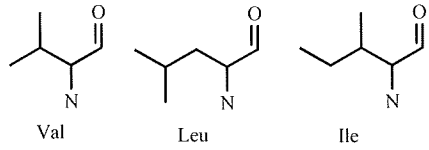

Fig. (3). Chemical structures of BCAAs.

PHARMACOLOGIC PROPERTIES OF BCAAs

Muscle-Protein Synthesis

BCAAs are mainly metabolized in skeletal muscle [96, 97] and serve as both a non-specific source of carbon for oxidation as fuel for the muscle [98] and as a precursor for the synthesis of other amino acids and proteins [99]. BCAAs have beneficial effects for decreasing exercise-induced muscle damage and promoting muscle-protein synthesis [100].

Immune System

Omission of valine, leucine, or isoleucine from the culture medium abolishes the proliferative activity of lymphocytes in response to phytohemagglutinin [101]. Administration of BCAAs improves maturation of monocyte-derived dendritic cells [102]. BCAAs also increase the number of liver-associated lymphocytes and augment lectin-dependent cellular cytototoxic activity in animals [103]. In human studies, treatment with BCAAs increases absolute lymphocyte count [104, 105] and improves phagocytic function of neutrophils, and natural killer activity of lymphocytes [106].

Ammonia Metabolism

Hyperammonemia is a common manifestation in patients with advanced liver cirrhosis and a causative factor for hepatic encephalopathy [107]. Hyperammonemia is caused by impaired hepatic ability to detoxify ammonia. BCAAs are source of glutamate, which detoxifies ammonia by glutamine synthesis in skeletal muscle and the brain [108, 109]. Therefore, BCAAs enhance detoxification ability of blood ammonia by incorporating ammonia in the process of glutamine production [110] and are currently used for the treatment for patients with hepatic encephalopathy.

EFFECTS OF BCAAs ON GLUCOSE METABOLISM

A couple of clinical studies have shown that protein-rich diets cause changes in plasma amino acid profile and improve insulin resistance, compared with normal diets having the same caloric content [111-114]. These findings suggest that amino acids have some pharmacologic effect on glucose metabolism. In contrast, plasma BCAAs levels are elevated in patients with obese or insulin resistance [115, 116]. Recently, Newgard *et al.* revealed the molecular mechanisms for BCAA-induced insulin resistance [116]. Thus, the oppose effects of BCAAs on glucose metabolism and insulin sensitization remain controversial. Herein, we summarize *in vitro*, *in vivo*, and clinical studies that examined the effects of BCAAs on glucose metabolism (Table **1**) and discuss the effects of BCAAs on glucose metabolism.

Effects of BCAAs on Insulin Secretion

Leucine is known to stimulate insulin secretion from pancreatic beta-cells [117, 118]. The mechanisms for leucine-induced insulin secretion are mediated by the metabolism of leucine *via* oxidative decarboxylation and allosteric activation of glutamate dehydrogenase in mitochondria by [119, 120]. Both acetyl-CoA and alpha-ketoglutarate are necessary as Kreb's cycle substrates to fully activate the beta-cell mitochondria, leading to insulin exocytosis induced by ATP [121]. In addition, some insulin signaling molecules such as AMP-activated protein kinase, pancreatic duodenal homeobox-1, and mTOR are involved in leucine-induced insulin secretion [121, 122]. Leucine-mediated activation of mTOR also contributes to enhance beta-cell function and the maintenance of beta-cell mass [121]. Thus, administration of leucine is a possible therapeutic strategy for diabetic patients with exhausted or decreased insulin secretion of beta-cells.

Effects of Intracellular Insulin Signaling Molecules

Among all amino acids, BCAAs have been demonstrated to modulate the insulin signaling cascade in muscle, adipose tissue, and liver (Table **1**). In high-fat diet mice, Zhang *et al.* showed that leucine supplementation improved glucose metabolism by reducing insulin resistance. Although leucine supplementation does not affect food intake, it increases uncoupling protein (UCP)-3 expression in skeletal muscle, brown adipose tissue, and white adipose tissue [123]. BCAAs, especially leucine, are reported to induce glucose uptake in primary rat muscle cells. Since the beneficial effect is inhibited by LY294002, a specific inhibitor of PI3K, leucine may stimulate insulin signaling pathways in skeletal muscle *via* preservation of PI3K activity [124, 125]. Similarly, in adipocytes from db/db mice, leucine augments the insulin-induced activation of the Akt/mTOR pathway [126]. Since insulin sensitivity is enhanced in p70 S6 kinase 1 (S6K1) $^{-/-}$ mice [127], mTOR/PI3K may be involved in the improvement in insulin resistance elicited by BCAAs.

The mTOR pathway is an important cascade for the regulation of insulin-stimulated glucose transport [128]. Nutrients including amino acids negatively affect insulin signaling through mTOR/S6K1 phosphorylation of IRS1 [129]. In a rat model of obesity-associated insulin resistance, BCAAs increase phospho-mTOR at Ser2448, phospho-S6K1 at

Table 1. Effects of BCAAs on Glucose Metabolism

	Cells/Animal models/Subjects	Amino acids	Administration term	Insulin resistance or glucose intolerance	Altered molecules or insulin effects	Reference
In vitro study	Primary rat muscle cells	Leucine	60 min	Improved	PI3K aPKC	[124]
	L6 skeletal muscle cells Primary rat muscle cells	BCAAs	180 min	Improved	PI3K aPKC	[125]
	Primary aciposytes from db/db mice	Leucine	80 min	Improved	Akt	[126]
In vivo study	High fat diet-male mice	Leucine	14-week	Improved	UCP-3 mTOR	[123]
	High fat diet-BCATm knockout mice	BCAAs	15-week	Improved	mTOR	[137]
	Exercise-trained rats	BCAAs	6-week	Improved	Hepatic and muscle glycogen contents	[213]
	Exercise-trained rats	BCAAs-enriched protein	4-week	Improved	Hepatic glucokinase	[131]
	CCl_4-treated rats	Leucine Isoleucine	60 min	Improved	GLUT1 GLUT4	[130]
	High fat diet-rats	BCAAs-enriched protein	6-week	Improved	N/A	[214]
	High fat diet-rats	BCAAs-enriched protein	6-week	Improved	N/A	[215]
	db/db mice	BCAAs	7-week	Improved	N/A	[216]
	Zucker fa/fa rats	Isoleucine	3-week	Improved	PI3K	[132]
	High fat diet-rats	BCAAs	16-week	Worsen	mTOR/S6K1, IRS1	[116]
Clinical study	Healthy male volunteers (n = 7)	Amino acids	120 min	Worsen	N/A	[142]
	Healthy elderly man (n = 30)	Leucine	12-week	No change	N/A	[143]
	Patients with type 2 diabetes mellitus (n =12)	High-protein diet	5-week	Improved	N/A	[217]
	Patients with chronic viral liver diseases (n = 12)	BCAAs	12-week	Improved	N/A	[33]
	Patients with HCV infection (n = 2)	BCAAs	6-week	Improved	N/A	[34]

Note. Abbreviation, aPKC, atypical protein kinase C; BCAAs, branched-chain amino acids; BCATm, mitochondrial branched chain amino acid aminotransferase; CCl_4, carbon tetrachloride; GLUT, glucose transporter; mTOR, mammalian target of rapamycin; N/A, not applicable; PI3K, phosphatidylinositol 3-kinase; UCP-3, uncoupling protein-3.

Thr389, and phospho-IRS1 at Ser302, leading to the development of insulin resistance [116]. However, in a rat model of liver cirrhosis, Nishitani *et al.* reported that administration of leucine and isoleucine decreases blood glucose levels by enhancing glucose uptake as a result of increased translocation of GLUT4 and GLUT1 to the plasma membrane of skeletal muscle [130]. Thus, effects of BCAAs on mTOR/S6K1 pathway are opposite and may be depend on the etiology of the insulin resistance.

Administration of BCAAs has also been reported to activate insulin signals in the liver. BCAAs-enriched protein activates hepatic glucokinase and results in increased hepatic glycogen contents [131]. Isoleucine also increases hepatic PI3K activity and improves insulin resistance in Zucker fa/fa rats, a model of severe insulin resistance [132]. Since insulin increases UCP-3 protein expression [133], translocation of GLUT4 and GLUT1 to the plasma membrane [134], glucokinase activity [135], and PI3K activity [136], BCAAs could improve insulin signals in various organs *via* various pathways.

Recently, She *et al.* clearly demonstrated an interaction between BCAAs and insulin resistance in mitochondrial

branched chain amino acid aminotransferase (BCATm) gene knockout mice. BCATm gene encodes the enzyme catalyzing the first step in peripheral BCAAs metabolism, and therefore, knockout of the BCATm gene leads to a significant elevation of plasma BCAAs levels. In $BCATm^{-/-}$ mice, fasting blood glucose and fasting serum insulin levels are decreased by 33% and 67%, respectively, and the homeostasis model assessment for insulin resistance (HOMA-IR) index, a marker of insulin resistance, is significantly lower compared to that of controls [137].

Taken together, BCAAs directly enhance insulin sensitivity by activating PI3K, Akt, and UCP-3. Since these pathways are down-stream of IRS, a target molecule for HCV-associated insulin resistance, BCAAs may be a candidate therapeutic agent for insulin resistance in patients with HCV infection.

Inhibition of Proteolysis

BCAAs inhibit proteolysis [96, 138, 139]. Indeed, BCAAs decrease expression levels of the genes involved in ATP-ubiquitin-dependent proteolysis [140, 141]. Since ubiquitin-proteasomal degradation of IRS1 and IRS2 is associated with HCV-associated insulin resistance, BCAAs may also contribute to improve insulin resistance by inhibiting degradation of IRS1 and IRS2. Inhibition of proteolysis is one of the possible mechanisms by which BCAAs combat insulin resistance.

Effects of BCAAs on Insulin Resistance

Clinical studies which evaluate effects of BCAA on glucose metabolism are summarized in Table **1**. No clinical studies show that BCAAs improve insulin resistance in healthy volunteers without apparent insulin resistance [142, 143]. However, we have recently demonstrated the beneficial effects of BCAAs administration on insulin sensitivity in chronic viral liver disease patients with insulin resistance [33]. Although body weight and plasma glucose concentration were unchanged, serum insulin levels and HOMA-IR index were significantly decreased after 60-days administration of BCAAs. Moreover, in two patients with HCV infection, BCAAs caused a decrease in both fasting insulin concentration and HOMA-IR index [34].

In contrast, elevated plasma concentration of BCAAs is reported in animal models of obesity and patients with insulin resistance [115, 116, 144]. Obesity-associated increase in BCAAs levels has been attributed to increased protein catabolism secondary to insulin resistance [145, 146] and impairment of BCAA metabolism [144]. Newgard *et al.* recently showed that BCAAs activate the mTOR/S6K1 pathway and phosphorylate multiple Ser residues of IRS, leading to the development of insulin resistance [116]. Although BCATm gene knockout mice showed increase in BCAA levels and reduction of insulin resistance [137], the absence of leucine-mediated suppression of proteolysis may contribute to the energy requirement for futile cycling of protein and subsequent reduction of insulin resistance [116]. Thus, the insulin-sensitizing effect of BCAAs remains controversial and may be only seen in patients with liver diseases, but not in obese patients with hyperinsulinemia or impairment of BCAA metabolism.

SAFETY OF BCAAs

Maple syrup urine disease is a genetic disorder impairing branched-chain alpha-keto acid dehydrogenase complex activity, resulting in the accumulation of BCAAs and branched-chain alpha-keto acids [147]. Patients with maple syrup urine disease often show severe neurological damage and mental retardation [148, 149], and therefore, tolerance limits for BCAAs have to be examined carefully.

Plasma BCAAs levels are decreased in patients with chronic liver diseases, especially liver cirrhosis [108, 150]. To detoxify ammonia, skeletal muscle uptakes and consumes BCAAs and subsequently increases clearance of BCAAs from plasma [108, 151]. Furthermore, BCAA catabolic enzymes are widely expressed throughout the body [152]. Thus, treatment with BCAAs is considered safe, as long as BCAA catabolism is at normal levels [153-155]. The prevalence of adverse effects of BCAAs is less than 15%, and major adverse effects are mild gastrointestinal symptoms such as abdominal distention, diarrhea, and constipation, but no neurological symptoms [156-162]. In general, adverse effects tend to disappear when treatment is discontinued.

PEDF

PEDF is a 50-kDa glycoprotein initially isolated from fetal human retinal pigment epithelial cells [163]. Sequence analysis of the 418 amino acids in human PEDF demonstrates a 27% identity to the serine protease inhibitor (serpin) prototype, α1-antitrypsin. PEDF has the typical serpin secondary and tertiary structure and belongs to the serpin superfamily [163-165]. Besides retinal pigment epithelial cells, PEDF is expressed in other parts of the eye (corneal epithelial cells and ciliary epithelium) [165], in other parts of the central nervous system (ependymal cells and motor neurons of the ventral horn) [166], and various cell types [166-169]. We have recently found that PEDF protein is expressed in human liver tissue, one of the target organs of insulin [170]. In addition, we have found that serum PEDF is elevated in patients with metabolic syndrome [167, 171]. Moreover, we have revealed an association between serum PEDF levels and HOMA-IR index in patients with HCV infection as shown in Fig. (**4**). These findings lead us to hypothesize that PEDF plays an important role in the development of HCV-associated insulin resistance. First, we review the structure-function relationship of PEDF and then, the molecular mechanisms by which PEDF improves insulin resistance.

STRUCTURE-FUNCTION RELATIONSHIP OF PEDF

Since HCV causes insulin resistance as well as the development of HCC, a hypervascular tumor, PEDF may have beneficial effects in HCV-related HCC patients *via* direct anti-tumor, anti-angiogenic and insulin-sensitizing properties. Here, we discuss which chemical structures could contribute to their multipotent functions.

Protein Structure Associated with Receptor-Binding

Receptor-binding activity of PEDF is preserved when the exposed loop is cleaved from its C-terminal end. PEDF pep-

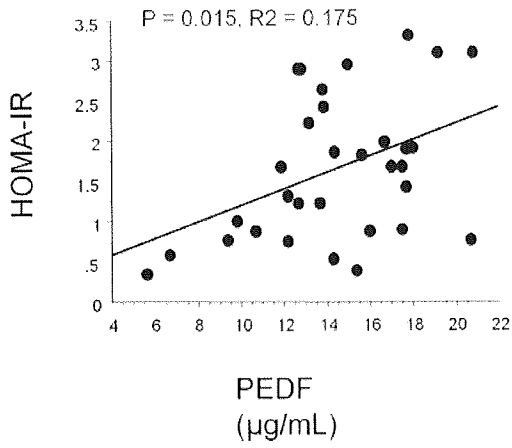

Fig. (4). An association between PEDF and HOMA-IR in patients with HCV infection. A positive correlation between serum PEDF levels and HOMA-IR index is seen in patients with HCV infection.

tides spanning residue position 32-380 and 44-121 have the ability to induce morphological differentiation and neurite outgrowth-inducing activity in human Y-79 retinoblastoma cells. Thus, N-terminal region of PEDF is a receptor-binding site [172] that has also been proven by the radioligand-binding assay and crystallization analysis [164, 173].

The heparin-binding site is in the loop region between sheet 2A and helix E [164, 174]. Site-directed mutagenesis examination revealed that Arg145, Lys146, and Arg148 are necessary for heparin binding [175]. Binding with heparin increases the proteolytic susceptibility of PEDF to trypsin and induces a conformational change in the vicinity of Lys178 [176]. Heparin facilitates the binding of PEDF to a receptor on the cell surface of retinoblastoma cells and structural change of the PEDF molecule is thought to be a mechanism for enhanced receptor binding [177]. These data indicate that a variety of PEDF functions are regulated by heparin-induced structural alteration as well as the N-terminal region.

Protein Structure Associated with Anti-Tumor Effect

PEDF contains putative hyaluronan (HA)-binding motifs. Becerra *et al.* examined the HA-binding region of PEDF by site-directed mutagenesis and identified $BXBX_2BX_2B$ and BX_3AB_2XB motifs as binding site HA (B, X, and A indicate basic amino acids, residues other than acidic amino acids, and negatively charged amino acids, respectively) [178]. These HA-binding proteins activate caspase-8, caspase-3, and poly (ADP-ribose) polymerase, which are triggers of apoptosis [179]. Moreover, the HA-binding proteins inhibit tumor growth on chorioallantoic membranes of chicken embryos and in nude mice xenograft models [179]. Thus, the HA-binding activity of PEDF may contribute to deposition in the extracellular matrix that subsequently has anti-tumor effects.

Protein Structure Associated with Anti-Angiogenic Effect

Collagen I-binding region is located opposite the heparin-binding region. In this region, the acidic amino acid residues Asp255, Asp257, and Asp299 are critical to collagen I-binding [175]. Mutation of the collagen I-binding region of PEDF is reported to cause tumor progression with neovascularization [180]. These data suggest that the collagen I-binding region of PEDF may play a crucial role in anti-angiogenesis.

MOLECULAR MECHANISMS UNDERLYING PEDF-MEDICATED IMPROVEMENT OF INSULIN RESISTANCE

PEDF is first characterized as a neurotrophic factor in 1989 [181]. Since then, a range of biological effects of PEDF has been disclosed. PEDF is now widely recognized as anti-tumor factor with an apoptotic and anti-angiogenic properties [163, 165, 182-186].

Inhibition of IkappaB Kinase (IKK) and NF-κB Activation

Under hyperglycemic, oxidative, and inflammatory conditions, advanced glycation end products (AGEs) progressively form and accumulate. Recently, we found that AGEs cause insulin resistance in Hep3B hepatoma cells where they activate Rac-1, and phosphorylate IRS1 at the Ser307 residue, and phosphorylate IKK [187, 188]. In insulin-exposed Hep3B cells, AGEs decrease tyrosine phosphorylation of IRS1 and inactivate PI3K [187, 188]. PEDF inhibits the harmful effects of AGEs on insulin sensitivity in Hep3B cells [187, 188]. Since serum levels of AGEs are higher in patients with HCV infection than in healthy subjects [189], PEDF could improve HCV-associated insulin resistance by inhibiting IKK as shown in Fig. (**5**).

As described above, activation of NF-κB and subsequent overproduction of inflammatory cytokines such as TNF-α play an important role in the development of HCV-associated insulin resistance [69, 72, 73, 84]. Zhang *et al* found that intravitreal injection of PEDF significantly reduced vascular hyper-permeability in rat models of diabetes and oxygen-induced retinopathy, correlating with the decreased levels of retinal inflammatory factors, including TNF-α [190]. In cultured retinal capillary endothelial cells, PEDF significantly decreases TNF-α expression under hypoxia [190]. Moreover, down-regulation of PEDF expression by siRNA results in significant increases of TNF-α secretion by retinal Müller cells [190]. Wang *et al.* found that PEDF inhibits high glucose-induced activation of NF-κB in cultured primary human renal mesangial cells. We also demonstrated that PEDF suppresses NF-κB activation in various types of cells including hepatoma cells [191-195]. These findings suggest that PEDF acts as an anti-inflammatory factor by blocking the NF-κB pathway and may combat HCV-associated insulin resistance as shown in Fig. (**5**).

Suppression of NADPH Oxidase Activity

HCV core protein and HCV NS3 stimulate ROS production through activation of NADPH oxidase in HCV transfected cells [74-78]. ROS activates NF-κB and up-regulates expression of TNF-α, which inactivates insulin-signaling cascade [79-83]. We have previously found that PEDF down-regulates mRNA levels of p22phox, Nox4, and gp91phox/Nox2, which are membrane components of

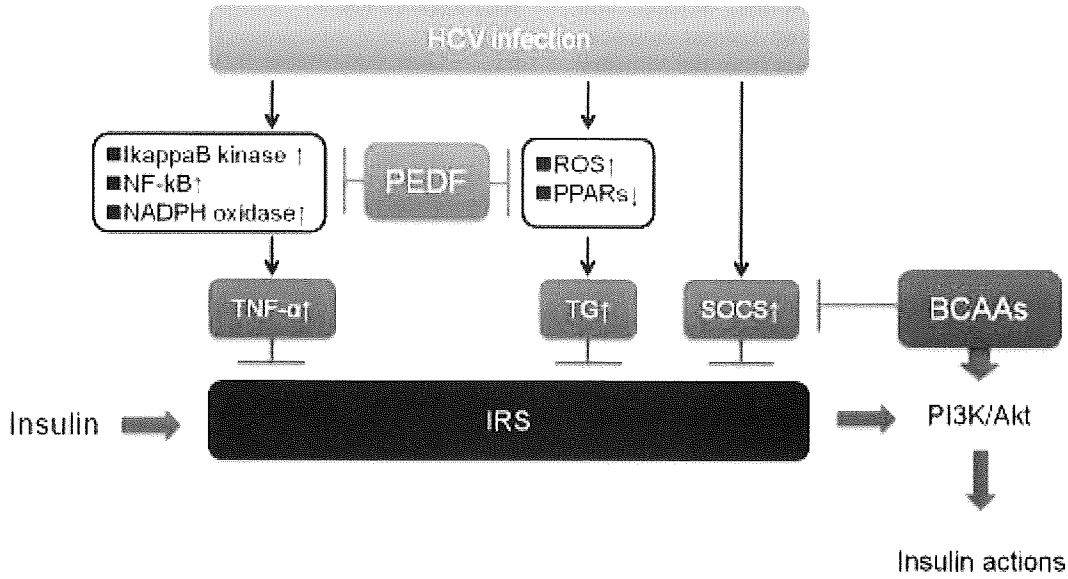

Fig. (5). Possible molecular mechanisms by which BCAAs and PEDF exert their insulin-sensitizing effects in patients with HCV infection. Abbreviations; BCAAs, branched-chain amino acids; HCV, hepatitis C virus; IRS, insulin receptor substrates; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-kappaB; PEDF, pigment epithelium-derived factor; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SOCS, suppressor of cytokine signaling; TG, triglyceride; TNF-α receptor 1.

NADPH oxidase, and decrease ROS derived from NADPH oxidase activity in endothelial cells [194]. We also found that PEDF inhibits ROS generation through suppression of NADPH oxidase activity *via* down-regulation of p22phox and gp91phox, and suppress the proliferation of smooth muscle cells induced by platelet-derived growth factor-BB [196]. Similarly, PEDF reduces ROS production *via* NADPH oxidase in T-cells and platelets [192, 194, 196-200]. Thus, PEDF may play a protective role against the development of HCV-associated insulin resistance *via* suppression of NADPH oxidase activity as shown in Fig. (**5**).

Reduction of Hepatic Triglyceride Accumulation *Via* Activation of PPARs

Hepatic triglyceride accumulation contributes to HCV-associated insulin resistance by decreasing PPARα and PPARγ expressions and subsequently various lipid metabolism-associated gene expressions [90, 92, 94]. Proteomic analysis revealed that PEDF is a potential regulator of lipid metabolism [201-203]. In fact, PEDF levels are correlated with severity of hepatic steatosis in both animals [204] and humans [171, 205]. In addition, hepatocytes isolated from PEDF null mice have about two-fold increase in triglyceride compared to hepatocytes from wild-type mice and hepatic triglyceride accumulation is an early event in livers from PEDF null mice [206]. Chung *et al.* found that PEDF is able to reduce the triglyceride content in a hepatoma cell line [207]. PEDF directly binds to PPARα and transfection of the PEDF gene in the presence of the PPARα/RXR heterodimer stimulates transcriptional activity of PPARα [207]. In addition, PEDF induces activation of PPARγ in various cell lines [207-212], PEDF may reduce hepatic triglyceride contents through induction of PPARγ not only in the liver, but also in other tissues thereby causing a redistribution of lipids. Thus, PEDF regulates hepatic lipid metabolism through modulation of PPARs activity and may ameliorate insulin resistance in patients with HCV infection as shown in Fig. (**5**).

CONCLUSION

We summarize the possible molecular mechanisms by which BCAAs and PEDF exert insulin-sensitizing properties in HCV-associated insulin resistance in Fig. (**5**). *In vitro-*, *in vivo-*, and human studies suggest that the following three pathways are responsible for HCV-associated insulin resistance in the liver; 1) proteasomal degradation of IRSs through up-regulation of SOCSs, 2) increased TNF-α expression, and 3) triglyceride accumulation. BCAAs improve insulin resistance by activation of insulin signaling molecules, which are down-stream of IRSs. In addition, BCAAs inhibit proteasomal degradation through down-regulation of ATP-ubiquitin-dependent proteolysis. Thus, BCAAs may improve insulin signaling pathways by two-different mechanisms. PEDF improves insulin resistance through down-regulation of TNF-α *via* suppression of IKK, NF-κB, and NADPH oxidase. PEDF also suppresses hepatic triglyceride accumulation by inhibition of ROS generation and activation of PPARs. Although direct evidence to show that BCAAs and PEDF improve insulin resistance in patients with HCV infection is still lacking, treatment with BCAAs and PEDF or pharmacological up-regulation of BCAAs and PEDF may be a promising therapeutic strategy for HCV-associated insulin resistance.

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ABBREVIATIONS

AGEs	=	advanced glycation end products
aPKC	=	atypical protein kinase C
BCAAs	=	branched-chain amino acids
BCATm	=	mitochondrial branched chain amino acid aminotransferase
CHOP	=	CCAAT/enhancer-binding protein homologous protein
ER	=	endoplasmic reticulum
ERK	=	extracellular signal-regulated kinase
GLUT	=	glucose transporter
HCC	=	hepatocellular carcinoma
HCV	=	hepatitis C virus
HNF-4	=	hepatocyte nuclear factor-4
HOMA-IR	=	the homeostasis model assessment for insulin resistance
IKK	=	inhibition of IkappaB kinase
IRS	=	insulin receptor substrates
MAPK	=	mitogen-activated protein kinase
mTOR	=	mammalian target of rapamycin
NADPH	=	nicotinamide adenine dinucleotide phosphate
NF-κB	=	nuclear factor-kappaB
NS	=	nonstructural protein
PDE3B	=	phosphodiesterase 3B
PDK	=	phosphoinositide-dependent kinase
PEDF	=	pigment epithelium-derived factor
PI3K	=	phosphatidylinositol 3-kinase
PPAR	=	peroxisome proliferator-activated receptor
ROS	=	reactive oxygen species
S6K1	=	S6 kinase 1
SOCS	=	suppressor of cytokine signaling
STAT	=	signal transducer and activator of transcription
TNF	=	tumor necrosis factor
TNFR1	=	TNF-α receptor 1
TG	=	triglyceride
Ub	=	ubiquitin
UCP-3	=	uncoupling protein-3
USF	=	upstream stimulatory factor
UTR	=	untranslated region

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