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Decreased expression of leptin receptors in non obese insulin resistant hepatitis C patients with high serum leptin level

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Abstract

Hepatitis C is a chronic and pestilent disease showing a strong association with diabetes. The mechanisms responsible for the pathogenesis of diabetes in association with hepatitis C remains to be identified. Leptin, a multifunctional cytokine secreted from adipocytes has been found to have high level in hepatitis C virus (HCV) infected patients. Leptin actions after remaining a mystery for many years are now finally beginning to yield some of its molecular secrets. Throughout this decade powerful new molecular approaches have revolutionized and broadened our molecular insight regarding the role of leptin in different diseases. The present study was designed to explore and substantiate the role of leptin in the pathogenesis of type 2 diabetes in hepatitis C infected patients. Serum leptin levels and insulin resistance in diagnosed cases of hepatitis C were measured and the expression of leptin and leptin receptors in peripheral blood mononuclear cells was ascertained by mRNA quantification. We discovered high serum leptin levels with decreased expression of leptin receptors in insulin resistant as compared with non insulin resistant HCV patients and normal individuals. This association of leptin resistance with insulin resistance in HCV patients through leptin receptor down regulation points towards a novel steps in mechanism implicated in disease pathogenesis which can lead to more targeted approach to therapy.

Keywords: *Leptin, Insulin resistance, Hepatitis C virus, Type 2 Diabetes*

Introduction

Hepatitis C and type 2 diabetes are two common disorders having a great impact on health world over. It has been frequently reported that type 2 diabetes (T2DM) has high prevalence among hepatitis C virus infected patients, and there is increasing evidence supporting the concept that hepatitis C virus infection is a risk factor for developing type 2 diabetes.[1] There are more significant chances of hepatitis C patients than control subjects to have insulin resistance along with high association of overall and liver-related mortality.[2]

The specific mechanisms by which hepatitis C virus leads to the development of type 2 diabetes are not clear but it appears that an increase in insulin resistance along with the overproduction of proinflammatory cytokines could play a key role.[3] Leptin is a hormone secreted from adipocytes which also acts as a cytokine.[4] It is known that insulin secretion and tissue responsiveness to insulin is regulated by serum leptin.[5] Leptin signals to the hypothalamus to regulate appetite and energy expenditure.[6] Leptin receptors are expressed in many peripheral tissues including pancreas, brain, heart, placenta, liver, skeletal muscle, lung, hematopoietic cells, and peripheral blood mononuclear cells (PBMCs).[7] Leptin acting through these receptors activate signaling pathways in peripheral blood mononuclear cells which then stimulate induction of cytokine and hence play role in immune function.[8] There is increasing evidence that innate immunity is involved in pathogenesis of type 2 diabetes by virtue of high levels of cytokine that produce insulin resistance.

Serum leptin levels significantly correlate with insulin resistance.[9] Nevertheless, the association of leptin and insulin resistance in patients with chronic viral hepatitis till now is not clear, both presence and absence of relationship between serum leptin and insulin levels have been observed in chronic viral hepatitis patients.[1]

In the present study, we determined the serum leptin levels and investigated the expression of leptin and leptin receptor (OB-R). The quantification of the two major leptin receptor isoforms (OB-Rl, OB-Rs) in peripheral blood mononuclear cells (PBMCs) was performed by real-time polymerase chain reaction (RT-PCR). In the present study, the role of leptin as a causative factor of type 2 diabetes mellitus in hepatitis C patients is corroborated and expression of leptin receptors is also investigated.

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Table 1: Primers of OB-Rl, OB-Rs and Leptin with their annealing temperature.

Gene	Forward Primer	Reverse Primer	Annealing Temperature
OB-R1	5-CAGAAGCCAGAAACGTTTG-3 spanning Exon-19 and exon -20	5-TCTCCCATGAGCTATTAGAG-3 Exon-20	58ºC
OB-RS	5'-TGTTGTGAATGTCTTGTGCC-3' Exon-6	5'-TGCTCCAGTCACTCCAGATTCC-3' Exon-8	57ºC
Leptin	5-CACACACGCAGTCAGTCTCC-3 spanning Exon-2and exon-3	5-TTAGAGAAGGCCAGCACGTG-3 Exon-3	55ºC

Patients and methods

Study subjects: A total of 40 human subjects of both genders were included in the study. The age group of the patients was $\geq$ 20 years while the upper limit of age bracket was kept at 50 years. Mean age of insulin-resistant patients was 38.75 while in non insulin-resistant it was 37.25. Patients with a body mass index (BMI) of $\leq$ 25 kg/m^2 were included in the study. The mean BMI of insulin-resistant group was 23.7 ± 1.46 kg /m2 and non insulin-resistant mean BMI was 22.88 ±1.45. All patients were polymerase chain reaction (PCR) positive HCV patients. Liver biopsy was not taken in these patients as these were all recently diagnosed patients of HCV without any complications and none of the patients had received any antiviral treatment. This exclusion was done to rule out any changes of insulin resistance and adipocytokines occurring under treatment. Previously known diabetics and pregnant women were excluded from the study. The control group consisted of 30 healthy individuals. All healthy individuals had normal alanine aminotransferase (ALT) values, tested negative for anti-HCV and were non –diabetic.

Data collection

The Ethical Committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College evaluated and approved the research. All patients fulfilling the inclusion criteria were elaborately apprised about the study and then their informed consent was taken. 10 ml of whole blood sample was collected in EDTA and serum tubes 5 ml in each tube, after keeping the subject fasting overnight for 10-12 hr and following parameters were measured in both groups. Fasting plasma glucose was checked by GOD/PAP enzymatic colorimetric method using linear chemicals S.L kit. Serum insulin was measured by chemiluminescence method using Immulite 1000. Their Homeostasis model assessment-Insulin resistance (HOMA-IR) was calculated as per formula of Mathew [10]. Those with insulin resistance were assigned to one group and with no insulin resistance to another group.

Serum leptin measurement

Serum leptin concentration was measured using a commercial sandwich enzyme-linked immunosorbent assay (ELISA; DRG Leptin EIA-2395) with a limit of detection of 1.0 ng/ml. The intra- and inter-assay coefficients of variation were 6.91% and 8.66%, respectively.

Total RNA isolation

Total cellular RNA was extracted from whole blood by using TRI Reagent BD-RNA isolation kit (Cat. No. TB126). Quality of isolated RNA was controlled spectrophotometrically (only the samples with 260/280nm ratio >1.8 were proceeded for further steps of analysis), the RNA integrity was controlled by agarose gel electrophoresis. The samples for RNA isolation were processed immediately after collection and stored at – 80^{o}C.

Real-time, Quantitative RT-PCR of (OB) m-RNA and Leptin

Quantification of leptin's mRNA was performed by real-time RT-PCR using the One step SYBR PrimeScript^{TM} RT-PCR kit (Takara, Bio Inc, Japan) according to the manufacturers' instructions.

The oligonucleotide primers used against OB-R1, OB-Rs and leptin gene along with their annealing temperatures and are given in table 1.

The PCR products are 168 base pairs (bp) for leptin, 387 bp for OB-Rl, and 394 bp for OB-Rs. Forward primer of leptin and OB-Rl was spanning last two exons in order to exclude any genomic DNA binding.

The instrument used for real-time RT-PCR was Smart Cycler® II System (Cepheid). The reagents used were prepared on ice and processed according to manufacturer protocol. Reaction tubes without RT enzyme were used as negative control. The reaction tubes were gently spinned down with Smart Cycler® specific centrifuge, and then the reaction was started after setting them onto Smart Cycler®. The housekeeping gene beta-actin was used as an internal positive control. For the analysis of data comparative Ct-value of leptin and leptin receptors in insulin resistant and non resistant patients were noted.

Use of Bioinformatic tools

In order to design an appropriate primer set we checked genomic DNA sequence of leptin and leptin receptors on UCSC (http://www.genome.ucsc.edu). Ensembl (http://www. http://www.ensembl.org) was used to recheck the gene sequence. Corresponding c-DNA and m-RNA sequences were also checked using both of above mentioned websites.

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2007 with analysis tool pack. Mean and SD for leptin and HOMA-IR, age, height, weight was determined. Percentages were presented for gender and sample size. Student's t test was applied to compare mean and standard deviation of serum leptin and Ct values of leptin receptors between insulin resistant and noninsulin resistant chronic hepatitis-C patients. $p<0.05$ was considered significant.

Table 2: Mean serum leptin and HOMA-IR values of insulin resistant and non insulin resistant HCV patients.

Statistical Parameters	Non Insulin Resistant		Insulin Resistant		t-test
	Mean	Std Dev	Mean	Std Dev	P< 0.001
Leptin Conc (ng/ml)	5.37	4.03	17.81	9.65	1.03E-06
HOMA-IR	1.44	0.46	5.12	2.43	1.98E-09

Table 3: Comparison of mean Ct values among control, noninsulin resistant and insulin resistant HCV patients.

Parameters	Control	Non insulin resistant	Insulin resistant
Leptin Ct	26.91	27.4	27.43
Ob-RI Ct	27.68	30.13	32
Ob-Rs Ct	26.05	28.21	29.57

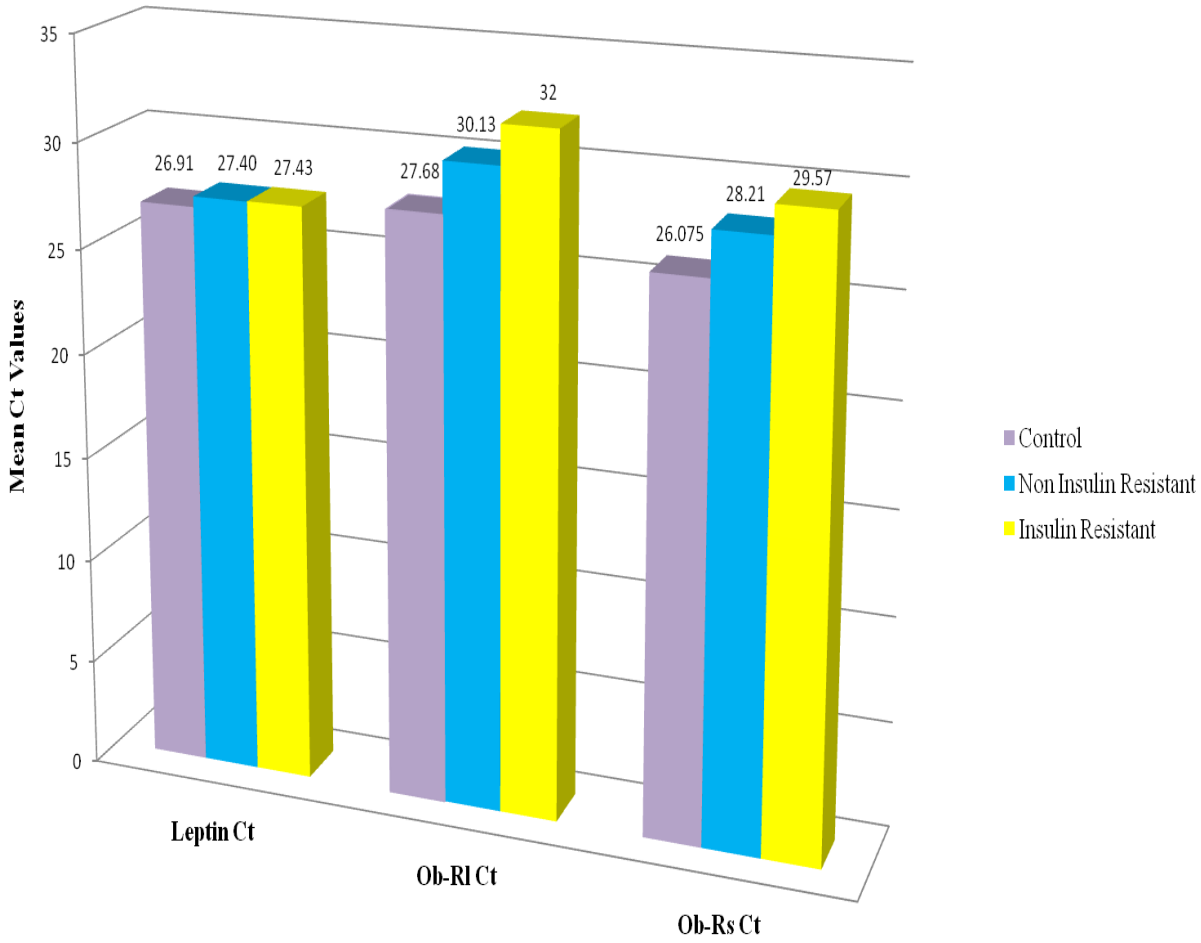

Figure 1: Mean Ct values of noninsulin resistant and insulin resistant HCV patients.

Results

HOMA-IR measurement

We divided our samples into two groups on the basis of insulin-resistance and non-resistance. Patients having HOMA-IR more than 2.5 were considered insulin-resistant and less than 2.5 were non insulin- resistant.[11-12] Out of 40 HCV patients 28 were found to be non insulin-resistant (mean 1.43 S.D ± 0.45) and 12 were insulin-resistant (mean 5.1 S.D±2.43)

Serum leptin measurements

Our results showed that serum leptin levels are higher in HCV patients as compared with healthy controls (4.08 ± 0.62 ng/ml) which are in accordance with a study which shows that serum leptin levels are higher in chronic hepatitis patients.[13] Serum leptin levels were increased, significantly more, in insulin-resistant HCV (17.81±9.65 ng/ml) patients compared with non-insulin resistant individuals (5.37±4.02 ng/ml) (Table 2). When both subgroups were taken together

females had higher serum leptin levels than males in the HCV patient group (females 15.65 ng/ml vs. males 7.2 ng/ml).

Relative Quantification of OB-Rl, OB-Rs, and leptin expression by on Real-Time RT-PCR in HCV Patients

By using quantitative real-time RT-PCR, we measured the mRNA levels of OB-Rl and OB-Rs in PBMCs of HCV patients. We found significantly lower expression levels of OB-Rl and OB-Rs in insulin-resistant HCV patients compared with non- insulin resistant individuals (Fig. 1).

There were differences among the mean levels of OB-Rl (31.97 ± 2.53) and OB-Rs (29.57± 4.7) mRNA in insulin-resistant and of OB-Rl (30.1 ± 2.72) and OB-Rs (28.21± 2.73) mRNA of non insulin- resistant HCV patients respectively (Table 3) (Fig. 1).

Our study has shown increased concentrations of serum leptin in insulin resistant as compared with non insulin resistant HCV patients but the expression of leptin in both the groups was the same. Also when we measured the expression of leptin receptors there was a down-regulation of long and short acting receptors of leptin.

Discussion

Throughout this decade, powerful new molecular approaches have broadened our molecular insight regarding the role of leptin in different diseases. Numerous studies have shown high serum leptin levels in hepatitis C patient.[1,13] The present study was designed to investigate the role of leptin as a missing link between hepatitis C and insulin resistance. In order to ascertain the link between these three, the expression of leptin and leptin receptors was noted. A previous, large cross-sectional study showed that it is three times more likely that persons with HCV infection will have T2DM than those without.[14] Another study showed that the site of insulin resistance was both peripheral and hepatic and not only there was basal increase of hepatic glucose production but it was less suppressed with insulin in the subjects with hepatitis C compared to controls.[15]

Serum leptin levels showed significant increase in insulin-resistant group (17.8±9.6 ng/ml) as compared with non insulin-resistant group (5.37±4.02 ng/ml) with Student's test, $P < 0.001$ in HCV patients that is suggestive of involvement of leptin with insulin resistance (Table 2). However, when we measured the expression of leptin using quantitative real-time RT-PCR there was no marked difference in both insulin resistant and non resistant groups as both non insulin resistant and insulin resistant groups showed a mean Ct value of 27.4 (Table 3).

In the present study, we also investigated the mRNA levels of OB-Rl and OB-Rs in patients with HCV which were significantly lower as compared with healthy individuals (Table 3). Interestingly, it has been noted that this expression was much lower in insulin resistant than non resistant group. This shows that high serum leptin levels in association with down regulation of its receptors may be linked with insulin resistance in HCV patients.

Insulin resistance results from defects at any level of the ligand-receptor-response pathway, including defects at the level of the insulin receptor or IRS molecules.[16-17] These defects can result from either reduced levels of signaling proteins, or modulation of their activity by phosphorylation.

The current data substantiate the fact that obesity, insulin resistance, and type 2 diabetes are closely linked by a crosstalk of the leptin and insulin-signaling pathways that impairs insulin action when serum leptin levels are high.[18] The insulin and leptin signaling pathways share some downstream molecules like Janus kinase 2 (Jak-2), insulin receptor substrates (Irs), phosphatidyl-inositol 3-kinase (PI3-K), protein kinase B (PKB), and mitogen-activated protein kinase (MAPK). These molecules can serve the role of mediators of the complex inhibitory crosstalk of leptin with the insulin-signaling chain.[19-20] Leptin pathway could interact with insulin signalling via serine/threonine inhibitory phosphorylation of IRS.[21]Previous data have suggested that leptin inhibits the insulin-signalling cascade in obese subjects by phosphorylation of Ser-318 in Irs1.[22] Thus leptin can contribute to the development of insulin resistance by its inhibitory effect on the insulin-signaling cascade.

In this study out of 40 HCV patients 12 were insulin resistant while other 28 were non resistant that shows 1:3 ratio. In fact, leptin stimulates glucose uptake[23] and resistance to its actions can prevent this important action. Fatty acid oxidation is enhanced by leptin[24] and it prevents lipid accumulation in both adipose and non adipose tissues. Any disruption to leptins action could promote ectopic lipid storage, which can further impair insulin sensitivity.[25]

The importance of adipocytokines has been highlighted by research which showed that high leptin levels also have a stimulatory effect on TNF-α which produces insulin resistance by being a negative regulator of insulin signal transduction.[26] Leptin is also able to control TNF-α production and activation by macrophages.[27]

Our findings are also suggestive of the involvement of leptin receptors in immune dysfunction in chronic HCV patients, as decreased OB-R expression results in disability of leptin to stimulate PBMCs, which can curtail the number of active immune cells. This is in accordance with previous studies, which showed hepatitis virus-induced down regulation of the immune system.[28]

Some studies have indicated that high levels of leptin are associated with insulin resistance, regardless of BMI.[29] In our research we had strict inclusive criteria of BMI less than 25 (non-obese) ranges. So our results are suggestive of leptin involvement with insulin resistance in HCV patients in spite of normal BMI. Results of current study substantiate the fact that the association between HCV and T2DM is more than just by chance and implication of leptin in its pathogenesis is now an admitted reality. Conclusively, the study is corroborative of the fact that leptin resistance results in insulin resistance as both the hormones share the same signaling pathway.

IR and T2DM not only stimulate the histological and clinical progression of chronic hepatitis C, but also decrease the response to antiviral therapy.[30] Hence a proper management of insulin resistance in patients with chronic hepatitis C could not only prevent disease complications but also prevent disease progression and increase rate to treatment.[31]

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