**Name of journal:** *World Journal of Gastroenterology*

**ESPS Manuscript NO: 7306**

**Columns: ORIGINAL ARTICLE**

**Hepatitis C virus NS5A promotes insulin resistance through IRS-1 serine phosphorylation and increased gluconeogenesis**

Parvaiz F *et al.* HCV NS5A modulates insulin signaling

Fahed Parvaiz, Sobia Manzoor, Jawed Iqbal, Mehuli Sarkar-Dutta, Muhammad Imran, Gulam Waris

**Fahed Parvaiz, Sobia Manzoor, Muhammad Imran,** Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology (NUST), Islamabad 44000, Pakistan

**Jawed Iqbal, Mehuli Sarkar-Dutta, Gulam Waris,** Department of Microbiology and Immunology, Rosalind Franklin University of Medicine and Science, Chicago, IL 60064, United States

**Author contributions**: Parvaiz F and Manzoor S designed the study and did the analysis; Parvaiz F performed most of the experiments and prepared the manuscript; Waris G provided technical support; Manzoor S and Waris G supervised the study and critically reviewed the manuscript; Iqbal J and Sarkar-Dutta M helped in performing Western blots and quantitative real-time polymerase chain reaction; Imran M helped in literature survey. All authors have read and approved the final version of the manuscript; Manzoor S is principal investigator and PhD supervisor of Parvaiz F.

**Correspondence to: Sobia Manzoor, PhD,** Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology, H-12, Islamabad 44000, Pakistan. [lcianunique@yahoo.com](mailto:lcianunique@yahoo.com)

**Telephone:** +92-51-90856147 **Fax:** +92-51-90856102

**Received:** November 12, 2013  **Revised:** February 21, 2014

**Accepted:** May 12, 2014

**Published online:**

**Abstract**

**AIM:** To investigate the mechanisms of insulin resistance in human hepatoma cells expressing hepatitis C virus (HCV) nonstructural protein 5A (NS5A).

**METHODS:** The human hepatoma cell line, Huh7 and Huh7.5 were infected with HCV or transiently-transfected with vector expressing HCV NS5A. The effect of HCV NS5A on the status of the critical players involved in insulin signaling was analyzed using real-time quantitative polymerase chain reaction and Western blot assays. Data was analyzed using Graph Pad Prism version 5.0 and 2-tail error bars represent SE of the data from three individual trials. *P*-value < 0.05 was regarded as statistically significant.

**RESULTS:** To investigate the effect of insulin treatment on the players involved in insulin signaling pathway, we analyzed the status of insulin receptor substrate-1 (IRS-1) phosphorylation in HCV infected cells or Huh7.5 cells transfected with HCV NS5A expression vector. Our results indicated that there is an increased phosphorylation of IRS-1 (Ser307) in HCV infected or NS5A transfected Huh7.5 compared to their respective controls. Furthermore, an increased phosphorylation of Akt (Ser473) was observed in HCV infected and NS5A transfected cells compared to their mock infected cells. In contrast, we observed decreased phosphorylation of Akt Thr308 phosphorylation in HCV NS5A transfected cells. These results suggest that Huh7.5 either infected with HCV or ectopically expressed with HCV NS5A alone has the potential to induce insulin resistance by the phosphorylation of IRS-1 at serine residue (Ser307) followed by decreased phosphorylation of Akt Thr308, Fox01 Ser256 and GSK3β Ser9, the downstream players of insulin signaling pathway. Furthermore, an increased expression of PECK and G6P, the molecules involved in gluconeogenesis, in HCV NS5A transfected cells was observed.

**CONCLUSION:** Taken together, our results suggest the role of HCV NS5A in the induction of insulin resistance by modulating the various cellular targets involved in insulin signaling pathway.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

**Key words**: Hepatitis C virus nonstructural protein 5A; Insulin resistance; Fox01; GSK3β; Gluconeogenesis

**Core tip:** The underlying molecular mechanisms of insulin resistance in response to hepatitis C virus (HCV) infection are poorly understood. Previous studies have demonstrated the effect of HCV core and envelop proteins in insulin signaling in human hepatocytes. However, the role of HCV nonstructural protein 5A (NS5A) in insulin resistance is not known. Our data clearly indicate the role of NS5A in insulin resistance through increased phosphorylation of IRS-1Ser307 and decreased phosphorylation of AktThr308, Fox01Ser256, and GSK3βSer9.

Parvaiz F, Manzoor S, Iqbal J, Sarkar-Dutta M, Imran M, Waris G.Hepatitis C virus

NS5A Promotes Insulin Resistance through IRS-1 Serine Phosphorylation and Increased Gluconeogenesis.*World J Gastroenterol* 2014; In press

**INTRODUCTION**

Hepatitis C virus (HCV) is a blood-borne pathogen, belongs to the family *Flaviviridae*. The HCV genome is a positive sense single stranded RNA molecule, which encodes a polyprotein that is cleaved by viral proteases and host cell signal peptidases into mature structural and non-structural proteins[1,2]. Chronic infection with HCV progresses into a number of pathological conditions including insulin resistance, fibrosis, steatosis, cirrhosis and ultimately hepatocellular carcinoma[3,4].

Insulin is the major anabolic hormone that has to utilize excessive glucose and maintain energy needs of the body. Insulin primarily performs this function by down regulating gluconeogenesis and glycogenolysis[4]. The primary targets of insulin action are skeletal muscles, cardiac muscles and liver[5]. In certain pathological conditions, insulin is vulnerable to perform its function and results in abnormal metabolic condition known as insulin resistance that refers to the complex array of metabolic disorders involving lipid deposition, enhanced fatty acids release and unfolded protein response[6-11]. One of the key downstream insulin signaling molecules is Akt that has been implicated in insulin resistance with the accumulation of diacylglycerols and ceramides[12]. In addition to this, ceramides may also accumulate an atypical isoform of protein kinase C (PKC) *i.e.,* PKC-zeta that sequester Akt and inhibits its function in normal insulin signaling[13,14].

In normal insulin signaling, insulin receptor substrate 1 (IRS-1) undergoes tyrosine phosphorylation and initiates a cascade of downstream signaling. However, there is impaired phosphorylation of IRS-1 in the obese patients with type 2 diabetes mellitus[15]. Studies also reveal that pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 are involved in insulin resistance via serine phosphorylation of IRS-1[16]. During insulin signaling, the insulin receptor has to bind with the adaptar protein, IRS and triggers the metabolic pathway effectively. In contrast, serine phosphorylation of IRS-1 leads to the degradation of IRS protein and hampers the insulin signaling pathway[4]. Akt is the downstream insulin signaling molecule which phosphorylates forkhead box protein 01 (Fox01) and glycogen synthase kinase-beta (GSK3β) and modulates insulin signaling[17,18]. Fox01 is the first direct downstream target of Akt and is involved in mediating hepatic glucose production via peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α)[19]. Fox01 regulates the expression of genes involved in glucose and lipid metabolism[20]. Fox01 undergoes post translational modifications including acetylation, ubiquitination and importantly phosphorylation to perform its stimulatory effect[18]. Fox01 has multiple phosphorylation sites but phosphorylation at serine 256 modulates its DNA binding activity and hampers normal metabolic pathways[21]. Downstream to the Akt/protein kinase B (PKB) signaling pathway also lies another important protein GSK3β that gets phosphorylated and inactivated in response to insulin, and suppresses key gluconeogenic genes *i.e.,* glucose-6-phosphatase (G6P) and phosphoenol pyruvate carboxykinase (PEPCK)[22,23]. Insulin regulates homeostasis by regulating two opposite pathways *i.e.,* glycolysis and gluconeogenesis. Gluconeogenesis involves the conversion of pyruvate back to glucose with the aid of PEPCK, G6P and several other enzymes[22-28]. In cyclic adenosine monophosphate (cAMP) axis, there is a cascade of genes that get activated and inactivated in response to the insulin mediated actions. Previous studies have shown that PGC-1α strongly upregulates gluconeogenic genes like CREB, PEPCK and G6P[29-31]. Akt inactivates PGC-1α and inhibits gluconeogenesis under normal metabolic conditions[32].

During the course of chronic HCV infection, the insulin signaling pathway is altered and the glucose can’t be metabolized properly with the concomitant increased transcriptional and translational expressions of gluconeogenic genes/proteins respectively[33,34]. So far little is known about the molecular mechanism behind the role of HCV in insulin resistance. Previously, HCV core protein has been shown to promote insulin resistance through serine phosphorylation of IRS-1 and modulating Akt signaling pathway[33,35].

In this study, we investigated the mechanism by which NS5A modulates key insulin signaling molecules such as IRS-1, Akt, Fox01 and GSK3β at the post translational level followed by the modulation of their target genes.

**MATERIALS AND METHODS**

***Antibodies and reagents***

Antibodies against IRS-1 (Ser307) and phospho-Akt (Ser473) were purchased from Calbiochem. Anti-phospho-Fox01 (Ser256) and anti-phospho-GSK3β (α/β) were purchased from Cell Signaling. Anti-phospho Akt (Thr308) and anti-actin were purchased from Santa Cruz Biotechnology and Sigma, respectively. Human recombinant insulin was purchased from Invitrogen. SYBR green master mix was purchased from Applied Biosystems.

***Cell lines***

Huh7 and Huh7.5 cell lines were grown in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin/mL and 100 μg of streptomycin/mL and cultured at 37°C under 5% CO2. Huh7.5 cell line was kindly provided by Dr. Charles Rice (Rockefeller University, NY) to Dr. Gulam Waris at Rosalind Franklin University of Medicine and Science (RFUMS), United States.

***HCV cell culture infection system***

HCV JFH-1 genomic RNA was *in vitro* transcribed and delivered into Huh7.5 cell line by electroporation or liposome mediated transfection. These cells were then plated in and passaged after 3 d. HCV infection in the cells and the corresponding cell culture supernatants was determined by quantitative real-time polymerase chain reaction (Applied Biosystems). The HCV cell culture supernatant was to infect naive Huh7.5 cells at appropriate dilutions (*moi* of 1). Cells were then incubated at 37 °C for about 5-6 h in 5% CO2 as previously described by[36]. The infectious JFH-1 construct was provided by Dr. Takaji Wakita to Dr. Gulam Waris at RFUMS, United States.

***Transient-transfection assays***

About 70% confluent cells in 60 mm petri dishes were transfected with HCV NS5A expressing plasmid using lipofectamine 2000 (Invitrogen, CA). The confluent cells were washed thrice with phosphate buffer saline to remove cell debris followed by treating with 100 nmol/L insulin for three hours before harvesting the transfected cells.

***Reverse transcription and quantitative real-time polymerase chain reaction***

Total cellular RNA was extracted from mock-infected, HCV infected and HCV NS5A transfected Huh7.5 cells using trizol (Invitrogen, CA). The extracted RNA was treated with DNase using RQ1 RNase-free DNase prior to cDNA synthesis. The cDNA was reverse-transcribed from 1 μg of total RNA using oligo(dT) primers according to the manufacturer’s protocol (Applied Biosystems, CA). Quantitative real-time polymerase chain reaction (RT-PCR) was carried out using SYBR green master mix and specific primer sets in triplicate. The 18s ribosomal RNA (18s rRNA) was used as an internal control. Amplification reactions were performed in 25 μL mix using RT-PCR reagent kit and the template RNA. Reactions were performed in 96-well spectroflourometric thermal cycle under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative transcript levels were calculated using ΔΔCt method as specified by the manufacturer. Primers were used as described previously (Table 1) [37,38].

***Western blot analysis***

Mock, HCV infected and NS5A transfected cells were harvested and cellular lysates were prepared by incubating the cells with RIPA buffer (50mM Tris Base pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.50% sodium deoxycholate, 0.10% SDS, 1mM orthovanadate, 1mM sodium formate and 10 μL/mL of protease inhibitor cocktail) on ice for 30. The lysates were subjected to SDS-PAGE followed by transfer to the nitrocellulose membrane in transfer buffer (25 mmol/L Tris, 192 mmol/L glycine and 20% methanol). The membranes were then incubated for 1hr in a blocking buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% Tween-20 and 5% non-fat dry milk). The membranes were then probed with respective primary antibody for 1hr at room temperature followed by washing thrice with blocking buffer without milk and then probed with respective secondary antibody for 1hr at room temperature. After doing an additional washing step, the membranes were visualized using Odyssey Infrared imaging system (Li-Cor Biosciences, Lincoln, NE). The expression of actin protein was used to determine protein loading control in each lane.

***Statistical analysis***

Data was analyzed using Graph Pad Prism version 5.0 and 2-tail error bars represent Standard error of mean (SE) of the data from three individual trials. *P*-value < 0.05 was regarded as statistically significant.

**RESULTS**

***Modulation of insulin signaling pathway in HCV infected hepatoma cells***

To investigate the effect of HCV upon insulin signaling pathway, we infected human hepatoma cells (Huh7 and Huh7.5) with HCV (JFH1) and confirmed the HCV infection with Western blot analysis of HCV NS3 as shown in Figure 1. Our results also showed that Huh7.5 cells are more susceptible to JFH-1 HCV infection than Huh7 cells.To examine the effect of HCV infection on the status of IRS-1 and Akt, the above cellular lysates were subjected to Western blot analysis. The results show an increased phosphorylation of IRS-1 (Ser307) and Akt (Ser473) in HCV infected Huh7 and Huh7.5 cells compared to their respective controls (Figure 1). Furthermore, the phosphorylation of IRS-1 and Akt was more in HCV infected Huh7.5 cells compared to HCV infected Huh7 cells (Figure 1, lane 4).

***HCV infected Huh7.5 cells show resistance towards insulin signaling***

Since we observed pronounced effect in Huh7.5 cells compared to Huh7 cells, therefore we selected Huh7.5 cells for further experiments. To examine the effect of HCV infection on the insulin signaling pathway in HCV infected Huh7.5 cells, uninfected and HCV infected Huh7.5 cells were incubated with insulin. The results show increased phosphorylation of IRS-1 (Ser307) in HCV infected cells which was reduced in HCV infected cells treated with insulin (Figure 2, lane 4). Furthermore, the phosphorylation of Akt (Ser473) was slightly enhanced in HCV infected cells treated with insulin. Collectively, these results suggest that HCV impairs insulin signaling via phosphorylation of IRS-1 (Ser307).

***HCV-NS5A alters phosphorylation level of IRS-1 (Ser307)***

To investigate the role of HCV NS5A in the induction of insulin resistance, Huh7.5 cells were transfected with HCV NS5A expression plasmid and the status of various cellular proteins involved in insulin signaling was examined. We observed increased phosphorylation of IRS-1 (Ser307) in NS5A transfected cells but not in cells treated with insulin (Figure 3). In addition, NS5A transfected cells showed an increased phosphorylation of Akt Ser473, compared to untransfected cells (Figure 3). In contrast, we observed decreased phosphorylation of AktThr308 in HCV NS5A transfected cells and it was not further affected upon insulin treatment (Figure 3). These results indicate the potential role of HCV NS5A in the modulation of insulin signaling pathway by the increased serine phosphorylation of IRS-1 and decreased phosphorylation of Akt Thr308.

***HCV-NS5A decreases the phosphorylation level of Fox01 (Ser256) and GSK-3β (Ser9)***

Fox01 is an important downstream target of insulin signaling pathway. The two important residues of Fox01 *i.e.,* Ser256 and Ser319, are known to be involved in nuclear exclusion of Fox01 and the regulation of the normal insulin mediated signaling[33]. We observed that HCV NS5A expressing cells show decreased phosphorylation of Fox01Ser256, and insulin treatment did not change the phosphorylation of Fox01, indicating that Fox01 may be involved in the modulation of subsequent downstream targets. In previous studies, insulin resistance has been linked with GSK3β signaling[17]. Similar to the Fox01Ser256, our results also show decreased phosphorylation of GSK3βSer9 in NS5A expressing cells indicating that active form of GSK3β favors gluconeogenesis in NS5A expressing cells (Figure 4).

***HCV infection promotes hepatic gluconeogenesis***

Downstream to the GSK3β are several gluconeogenic genes and transcription factors that are involved in gluconeogenesis. The rate limiting step of gluconeogenesis is controlled by phosphoenol pyruvate kinase (PEPCK) gene. Therefore, we first examined the transcriptional level of PEPCK in HCV infected cells. The results show significant increased expression of PEPCK indicating that HCV infected cells potentially enhances gluconeogenesis (Figure 5A). In addition, cAMP response element binding protein (CREB) is another important transcription factor that regulates the transcriptional activity of PEPCK. Our results showed significant increased expression of CREB in the HCV infected cells (Figure 5A). Furthermore, TNF-α, has also been linked to various HCV induced metabolic disorders. This prompted us to examine the transcriptional level of TNF-α in HCV infected cell line. The results showed a significant increased expression of TNF-α (Figure 5A), indicating the fact that this proinflammatory cytokine might have important role in the insulin resistance mechanism.

***NS5A promotes gluconeogenesis through transcriptional upregulation of PEPCK and G6P***

In order to investigate the role of HCV-NS5A in the upregulation of gluconeogenesis, we transfected Huh7.5 cells with NS5A and observed that there was significant increased expression of PEPCK. The ultimate downstream step of gluconeogenesis *i.e.,* conversion of glucose-6-phosphate to glucose, is governed by G6P. Similarly, the results indicated a significant increased transcriptional expression of G6P in HCV-NS5A transfected cells (as shown in the Figure 5B). These results suggest that gluconeogenic pathway is increased in NS5A expressing cells.

***Model of HCV NS5A induced insulin resistance***

The Model of HCV-NS5A induced insulin resistance is shown in the Figure 6.

**DISCUSSION**

Insulin resistance is a multifaceted disorder that involves modulation at transcriptional and translational level of various genes. Hepatic gluconeogenesis is the core phenomenon that provokes insulin resistance. Our data suggest that HCV infection or ectopic expression of HCV NS5A increases hepatic gluconeogenesis as well as modulates phosphorylation status of various cellular proteins required for the induction of insulin resistance. Normal insulin signaling involves the binding of the insulin to insulin receptor and promotes tyrosine phosphorylation of IRS. Downstream to the IRS-1 is the Akt, Fox01 and GSK3β that gets differentially phosphorylated and metabolize glucose and favor homeostasis[5,17,18]. Our results suggest that HCV infection in the hepatoma cell lines favors serine phosphorylation of IRS-1 (Ser307) that is required for the insulin resistance. Furthermore, we observed increased phosphorylation of Akt Ser473 and decreased phosphorylation of Akt Thr308 in HCV NS5A transfected cells. These results are consistent with the previous studies where Akt Thr308 but not Akt Ser473 phosphorylation plays an important role in insulin resistance process[33,35,41].

Fox01 is an important downstream insulin signaling molecule of Akt and has been implicated in the modulation of transcriptional regulation of various genes including PEPCK, G6P *etc.*, involved in gluconeogenesis[39]. Fox01 Ser256 is an important phosphorylation site that modulates its DNA binding ability, inhibits Fox01 nuclear translocation to the cytoplasm and modulates the metabolic gene expression[33]. Furthermore, GSK3β is another important downstream insulin signaling molecule that gets hypophosphorylated and becomes activated which, in turns, selectively upregulates the gluconeogenic gene expression and inhibitors of GSK3β can selectively downregulate the transcriptional expression of these gluconeogenic genes[22,40]. Previous studies have shown that decreased phosphorylation of GSK3β leads to its activation and favors gluconeogenesis[18,41]. To further discern the effect of HCV-NS5A on downstream targets of insulin signaling pathway, phosphorylation status of Fox01 Ser256 was examined in this study. Our results suggested that there was decreased phosphorylation level of Fox01 Ser256 in the HCV NS5A transfected cell line compared to its controls. Furthermore, the same pattern of decreased phosphorylation of GSK3β was observed in the transfected cell line

Taken together, our data reveal that HCV NS5A is potentially able to modulate normal insulin signaling pathway at various cellular points and favors the gluconeogenic pathway.

PEPCK is the rate limiting step of gluconeogenesis as it dictates the fate of gluconeogenesis by converting oxaloacetate to phosphoenol pyruvate and is greatly linked with the phenomenon of insulin resistance. CREB is the main transcription factor that governs transcriptional level of PEPCK and favors gluconeogenesis. In addition, some recent data has linked obesity and insulin resistance with the upregulation of TNFα[42-45]. Our data elucidates that HCV NS5A has strong role in the enhancement of gluconeogenesis by the way of increased expression of its key gluconeogenic genes *i.e.,* PEPCK and G6P with the concomitant increased expression of its transcription factors and inflammatory cytokine CREB and TNF-α respectively.

In this study, we have demonstrated that HCV NS5A favors serine phosphorylation of IRS-1, which is critically involved in the modulation of downstream insulin signaling pathway (Figure 6). Furthermore, downstream targets of insulin signaling pathway (Akt Thr308, Fox01 and GSK3β) undergo decreased level of serine phosphorylation that is involved in the inhibition of glycogen synthesis and favors gluconeogenesis, thereby imparts its role in the induction of hepatic insulin resistance. Hence, we characterized a mechanism through which HCV NS5A can modulate various cellular check points and leads toward hepatic insulin resistance.

**ACKNOWLEDGEMENTS**

We are thankful to Dr. Takaji Wakita (NIID, Tokyo, Japan) and Dr. Charles Rice (Rockfeller University, United States) for the generous gift of HCV genotype 2a (JFH1) and Huh7.5 cell line to Dr. Gulam Waris at RFUMS. We are thankful to Dr. Lance Presser and Steven McRae (RFUMS) for providing technical support. We are also thankful to Dr. Gulam Waris (RFUMS, United States) as this work was conducted in his lab by Fahed Parvaiz during the fellowship program.

**COMMENTS**

***Background***

Hepatitis C virus (HCV) is a lethal blood borne pathogen targeting hepatocytes and causes chronic infection in majority of the infected individuals. Some studies reveal that chronic HCV infection attenuates insulin signaling pathway which can lead to glucose intolerance and the development of insulin resistance. So far, HCV core protein has been clearly shown to induce insulin resistance through the modulation of signaling pathways and upregulation of gluconeogenesis. Up till now, there is no conclusive study that reveals the potential of HCV non structural protein 5A (NS5A) in the induction of insulin resistance through the modulation of insulin receptor substrate-1 (IRS-1) protein.

***Research frontiers***

HCV targets hepatocytes where various HCV proteins get replicated and favor various pathological conditions like insulin resistance, a step towards type 2 diabetes mellitus. The hotspot of this research article is the identification of HCV NS5A as a potential candidate for the development of insulin resistance. This study reveals that HCV NS5A modulates insulin signaling pathway leading to increase gluconeogenesis.

***Innovations and breakthroughs***

The underlying molecular mechanisms of insulin resistance in response to HCV infection are poorly understood. Previous studies have demonstrated the effect of HCV core and envelop proteins in insulin signaling in human hepatocytes. However, the role of HCV NS5A in insulin resistance is not known. Their data clearly indicate the role of NS5A in insulin resistance through increased phosphorylation of IRS-1Ser307 and decreased phosphorylation of AktThr308, Fox01Ser256, and GSK3Ser9.

***Applications***

This study results suggests that HCV NS5A has multiple cellular targets that should be prevented in order to reduce disease pathogenesis. Furthermore, it highlights the fact that HCV NS5A specific inhibitors should be synthesized that can reduce the chance of disease progression and morbidity rate.

***Terminology***

Insulin resistance: Insulin resistance is a complicated metabolic disorder that refers to the pre-diabetic phase with the modulation of insulin signaling at various cellular checkpoints like insulin receptors, IRS and impairment of homeostasis; Gluconeogenesis: Synthesis of glucose from non-glucose moieties; Fibrosis: A pathological condition in which excessive fats are deposited over liver; Cirrhosis: A pathological condition in which liver shrinks.

***Peer review***

This paper describes the influence HCV NS5A on serine phosphorylation of: insulin receptor substrate-1; Fox01 and GSK-3β and key gluconeogenic enzyme genes mRNA level. The paper reports a potentially interesting and an important study.

**REFERENCES**

1 **Bartenschlager R**, Lohmann V. Replication of hepatitis C virus. *J Gen Virol* 2000; **81**: 1631-1648 [PMID: 10859368]

2 **Reed KE**, Rice CM. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Curr Top Microbiol Immunol* 2000; **242**: 55-84 [PMID: 10592656]

3 **Sheikh MY**, Choi J, Qadri I, Friedman JE, Sanyal AJ. Hepatitis C virus infection: molecular pathways to metabolic syndrome. *Hepatology* 2008; **47**: 2127-2133 [PMID: 18446789 DOI: 10.1002/hep.22269]

4 **Sesti G**. Pathophysiology of insulin resistance. *Best Pract Res Clin Endocrinol Metab* 2006; **20**: 665-679 [PMID: 17161338 DOI: 10.1016/j.beem.2006.09.007]

5 **DeFronzo RA**. Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 1988; **37**: 667-687 [PMID: 3289989]

6 **Friedman J**. Fat in all the wrong places. *Nature* 2002; **415**: 268-269 [PMID: 11796987 DOI: 10.1038/415268a]

7 **Liu L**, Zhang Y, Chen N, Shi X, Tsang B, Yu YH. Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *J Clin Invest* 2007; **117**: 1679-1689 [PMID: 17510710 DOI: 10.1172/JCI30565]

8 **Ranganathan G**, Unal R, Pokrovskaya I, Yao-Borengasser A, Phanavanh B, Lecka-Czernik B, Rasouli N, Kern PA. The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment. *J Lipid Res* 2006; **47**: 2444-2450 [PMID: 16894240 DOI: 10.1194/jlr.M600248-JLR200]

9 **Turpin SM**, Hoy AJ, Brown RD, Rudaz CG, Honeyman J, Matzaris M, Watt MJ. Adipose triacylglycerol lipase is a major regulator of hepatic lipid metabolism but not insulin sensitivity in mice. *Diabetologia* 2011; **54**: 146-156 [PMID: 20842343 DOI: 10.1007/s00125-010-1895-5]

10 **Unger RH**. Lipotoxic diseases. *Annu Rev Med* 2002; **53**: 319-336 [PMID: 11818477 DOI: 0.1146/annurev.med.53.082901.104057]

11 **Samuel VT**, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. *Cell* 2012; **148**: 852-871 [PMID: 22385956 DOI: 10.1016/j.cell.2012.02.017]

12 **Powell DJ**, Hajduch E, Kular G, Hundal HS. Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. *Mol Cell Biol* 2003; **23**: 7794-7808 [PMID: 14560023 DOI: 10.1128/MCB.23.21.7794-7808.2003]

13 **Blouin CM**, Prado C, Takane KK, Lasnier F, Garcia-Ocana A, Ferré P, Dugail I, Hajduch E. Plasma membrane subdomain compartmentalization contributes to distinct mechanisms of ceramide action on insulin signaling. *Diabetes* 2010; **59**: 600-610 [PMID: 19959757 DOI: 10.2337/db09-0897]

14 **Yen CL**, Stone SJ, Koliwad S, Harris C, Farese RV. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J Lipid Res* 2008; **49**: 2283-2301 [PMID: 18757836 DOI: 10.1194/jlr.R800018-JLR200]

15 **Goodyear LJ**, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 1995; **95**: 2195-2204 [PMID: 7537758 DOI: 10.1172/JCI117909]

16 **Hotamisligil GS**, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 1996; **271**: 665-668 [PMID: 8571133]

17 **Cross DA**, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; **378**: 785-789 [PMID: 8524413 DOI: 10.1038/378785a0]

18 **Brunet A**, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; **303**: 2011-2015 [PMID: 14976264 DOI: 10.1126/science.1094637]

19 **Puigserver P**, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, Spiegelman BM. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* 2003; **423**: 550-555 [PMID: 12754525]

20 **Matsumoto M**, Han S, Kitamura T, Accili D. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. *J Clin Invest* 2006; **116**: 2464-2472 [PMID: 16906224 DOI: 10.1172/JCI27047]

21 **Zhao X**, Gan L, Pan H, Kan D, Majeski M, Adam SA, Unterman TG. Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. *Biochem J* 2004; **378**: 839-849 [PMID: 14664696 DOI: 10.1042/BJ20031450]

22 **Lochhead PA**, Coghlan M, Rice SQ, Sutherland C. Inhibition of GSK-3 selectively reduces glucose-6-phosphatase and phosphatase and phosphoenolypyruvate carboxykinase gene expression. *Diabetes* 2001; **50**: 937-946 [PMID: 11334436]

23 **Coghlan MP**, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ, Rausch OL, Murphy GJ, Carter PS, Roxbee Cox L, Mills D, Brown MJ, Haigh D, Ward RW, Smith DG, Murray KJ, Reith AD, Holder JC. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* 2000; **7**: 793-803 [PMID: 11033082]

24 **Yabaluri N**, Bashyam MD. Hormonal regulation of gluconeogenic gene transcription in the liver. *J Biosci* 2010; **35**: 473-484 [PMID: 20826956 DOI: 10.1007/s12038-010-0052-0]

25 **O'Brien RM**, Granner DK. PEPCK gene as model of inhibitory effects of insulin on gene transcription. *Diabetes Care* 1990; **13**: 327-339 [PMID: 2407480]

26 **Sutherland C**, O'Brien RM, Granner DK. New connections in the regulation of PEPCK gene expression by insulin. *Philos Trans R Soc Lond B Biol Sci* 1996; **351**: 191-199 [PMID: 8650266]

27 **Foster JD**, Pederson BA, Nordlie RC. Glucose-6-phosphatase structure, regulation, and function: an update. *Proc Soc Exp Biol Med* 1997; **215**: 314-332 [PMID: 9270716]

28 **Lange AJ**, Argaud D, el-Maghrabi MR, Pan W, Maitra SR, Pilkis SJ. Isolation of a cDNA for the catalytic subunit of rat liver glucose-6-phosphatase: regulation of gene expression in FAO hepatoma cells by insulin, dexamethasone and cAMP. *Biochem Biophys Res Commun* 1994; **201**: 302-309 [PMID: 8198588 DOI: 10.1006/bbrc.1994.1702]

29 **Herzig S**, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, Montminy M. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* 2001; **413**: 179-183 [PMID: 11557984 DOI: 10.1038/35093131]

30 **Yoon JC**, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman BM. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 2001; **413**: 131-138 [PMID: 11557972 DOI: 10.1038/35093050]

31 **Herzog B**, Hall RK, Wang XL, Waltner-Law M, Granner DK. Peroxisome proliferator-activated receptor gamma coactivator-1alpha, as a transcription amplifier, is not essential for basal and hormone-induced phosphoenolpyruvate carboxykinase gene expression. *Mol Endocrinol* 2004; **18**: 807-819 [PMID: 15044597 DOI: 10.1210/me.2003-0384]

32 **Li X**, Monks B, Ge Q, Birnbaum MJ. Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator. *Nature* 2007; **447**: 1012-1016 [PMID: 17554339 DOI: 10.1038/nature05861]

33 **Banerjee A**, Meyer K, Mazumdar B, Ray RB, Ray R. Hepatitis C virus differentially modulates activation of forkhead transcription factors and insulin-induced metabolic gene expression. *J Virol* 2010; **84**: 5936-5946 [PMID: 20357092 DOI: 10.1128/JVI.02344-09]

34 **Parvaiz F**, Manzoor S, Tariq H, Javed F, Fatima K, Qadri I. Hepatitis C virus infection: molecular pathways to insulin resistance. *Virol J* 2011; **8**: 474 [PMID: 22008087 DOI: 10.1186/1743-422X-8-474]

35 **Banerjee S**, Saito K, Ait-Goughoulte M, Meyer K, Ray RB, Ray R. Hepatitis C virus core protein upregulates serine phosphorylation of insulin receptor substrate-1 and impairs the downstream akt/protein kinase B signaling pathway for insulin resistance. *J Virol* 2008; **82**: 2606-2612 [PMID: 18160431 DOI: 10.1128/JVI.01672-07]

36 **Burdette D**, Olivarez M, Waris G. Activation of transcription factor Nrf2 by hepatitis C virus induces the cell-survival pathway. *J Gen Virol* 2010; **91**: 681-690 [PMID: 19889935 DOI: 10.1099/vir.0.014340-0]

37 **Cascio S**, Zhang L, Finn OJ. MUC1 protein expression in tumor cells regulates transcription of proinflammatory cytokines by forming a complex with nuclear factor-κB p65 and binding to cytokine promoters: importance of extracellular domain. *J Biol Chem* 2011; **286**: 42248-42256 [PMID: 22021035 DOI: 10.1074/jbc.M111.297630]

38 **Luo Z**, Zhang Y, Li F, He J, Ding H, Yan L, Cheng H. Resistin induces insulin resistance by both AMPK-dependent and AMPK-independent mechanisms in HepG2 cells. *Endocrine* 2009; **36**: 60-69 [PMID: 19440859 DOI: 10.1007/s12020-009-9198-7]

39 **Kamagate A**, Qu S, Perdomo G, Su D, Kim DH, Slusher S, Meseck M, Dong HH. FoxO1 mediates insulin-dependent regulation of hepatic VLDL production in mice. *J Clin Invest* 2008; **118**: 2347-2364 [PMID: 18497885 DOI: 10.1172/JCI32914.]

40 **Bernsmeier C**, Heim MH. Insulin resistance in chronic hepatitis C: mechanisms and clinical relevance. *Swiss Med Wkly* 2009; **139**: 678-684 [PMID: 20047129 DOI: smw-12765]

41 **Hsieh MJ**, Lan KP, Liu HY, Zhang XZ, Lin YF, Chen TY, Chiou HL. Hepatitis C virus E2 protein involve in insulin resistance through an impairment of Akt/PKB and GSK3β signaling in hepatocytes. *BMC Gastroenterol* 2012; **12**: 74 [PMID: 22721429 DOI: 10.1186/1471-230X-12-74]

42 **Pandey AK**, Bhardwaj V, Datta M. Tumour necrosis factor-alpha attenuates insulin action on phosphoenolpyruvate carboxykinase gene expression and gluconeogenesis by altering the cellular localization of Foxa2 in HepG2 cells. *FEBS J* 2009; **276**: 3757-3769 [PMID: 19769745 DOI: 10.1111/j.1742-4658.2009.07091.x]

43 **Hanson RW**, Reshef L. Regulation of phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* 1997; **66**: 581-611 [PMID: 9242918 DOI: 10.1146/annurev.biochem.66.1.581]

44 **Miyamoto H**, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, Miyamura T, Koike K, Matsuura Y. Involvement of the PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein. *J Virol* 2007; **81**: 1727-1735 [PMID: 17135326 DOI: 10.1128/JVI.01683-06]

45 **Lim JH**, Lee HJ, Ho Jung M, Song J. Coupling mitochondrial dysfunction to endoplasmic reticulum stress response: a molecular mechanism leading to hepatic insulin resistance. *Cell Signal* 2009; **21**: 169-177 [PMID: 18950706 DOI: 10.1016/j.cellsig.2008.10.004]

**P-Reviewers:** Gillessen A, Haidara M, Lehtonen SH, Swierczynski JT **S-Editor:** Gou SX  **L-Editor: E-Editor:**

**Figure legends**



**Huh7**

**Huh7+HCV**

**Huh7.5**

**Huh7.5+HCV**



**NS3 (70 kDa)**

**Akt (Ser473)**

**(65 kDa)**

**Actin (42 kDa)**



**IRS-1 (Ser307)**

**(165 kDa)**

**1 2 3 4**

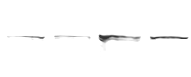
**Figure 1** **Hepatitis C virus infection modulates insulin signaling in hepatitis C virus infected human hepatoma cells.** Cellular lysates were made from mock and hepatitis C virus (HCV) infected Huh7 and Huh7.5 cells. Equal amount of cellular lysates were subjected to Western blot assay using p-IRS-1 Ser307and p-Akt Ser473. HCV NS3 protein expression represents the level of HCV infection. Cellular actin was used as an internal control to verify protein loading in each lane. IRS: Insulin receptor substrate; NS: Nonstructural protein.

**IRS-1 (pSer307)**

**(165kDa)**

**Akt (pSer473)**

**(65kDa)**



**Actin (42kDa)**

**Insulin (100nM) ― + ― +**

**Huh7.5**

**HCV (JFH-1)**

**1 2 3 4**

**Figure 2** **The status of p-Ser307 insulin receptor substrate-1 and p-Ser473 Akt phosphorylation in hepatitis C virus infected hepatoma cell line upon insulin treatment.** Total cellular lysates were prepared from hepatitis C virus (HCV) infected and mock infected Huh7.5 cells that were treated or untreated with insulin (100 nmol/L). Equal amount of cellular lysates were subjected to Western blot assay using anti-p-IRS-1 Ser307 and anti-p-Akt Ser473. Cellular actin was used as protein loading control in each lane. IRS: Insulin receptor substrate.



**Insulin (100nM) ― + ― +**

**Huh7.5**

**HCV NS5A**

**IRS-1 (pSer307)**

**(165kDa)**

**Akt (pSer473)**

**(65kDa)**

**Actin (42kDa)**

**1 2 3 4**



**Akt (Thr308)**

**(65kDa)**

**Actin**

**(42kDa)**

**Figure 3** **Hepatitis C virus nonstructural protein 5A modulates phosphorylation level of key insulin signaling molecules.** Untransfected and nonstructural protein 5A (NS5A) transfected cells were incubated with insulin (100 nmol/L) for 3h. Equal amount of cellular lysates were subjected to Western blot assay using anti-p-Akt Ser473, anti-p-Akt Thr308 and anti-p-IRS-1 Ser307. Cellular actin was used as an internal control to verify protein loading in each lane. IRS: Insulin receptor substrate.

**Actin (42kDa)**

**FoxO1 (Ser256)**

**(82 kDa)**

**p Ser α21**

**pSer β9**

**(51 kDa)**

**(47 kDa)**



}

**GSK-3**

**Insulin (100nM) ― + ― +**

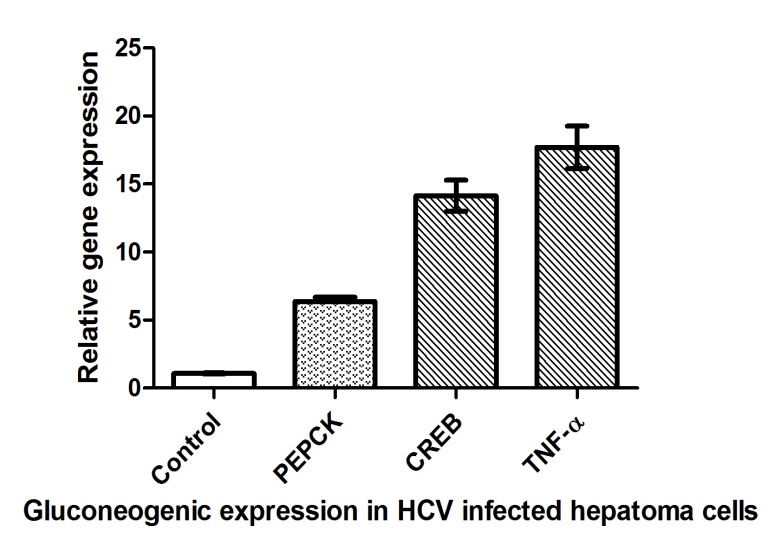
**Huh7.5**

**HCV NS5A**

**1 2 3 4**

**Figure 4 Effect of hepatitis C virus nonstructural protein 5A on the phosphorylation levels of Fox01 Ser256 and GSK-3β Ser9.** Using the cellular lysates from nonstructural protein 5A (NS5A) transfected cell line and the controlled treated hepatoma cell line, Western blot assay was performed using anti-p-GSK3 Ser (α21/ β9) and anti-p-Fox01 Ser256. HCV: Hepatitis C virus.

A)

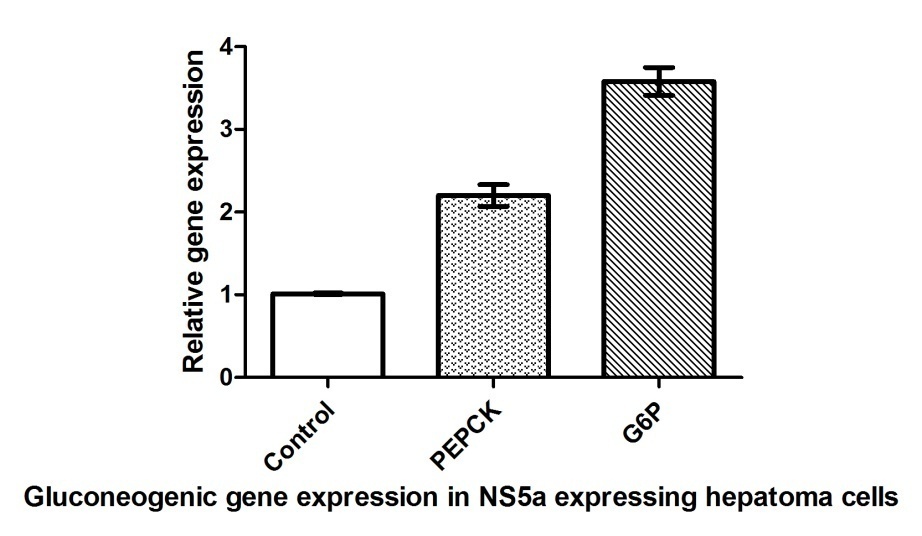


a

a

a

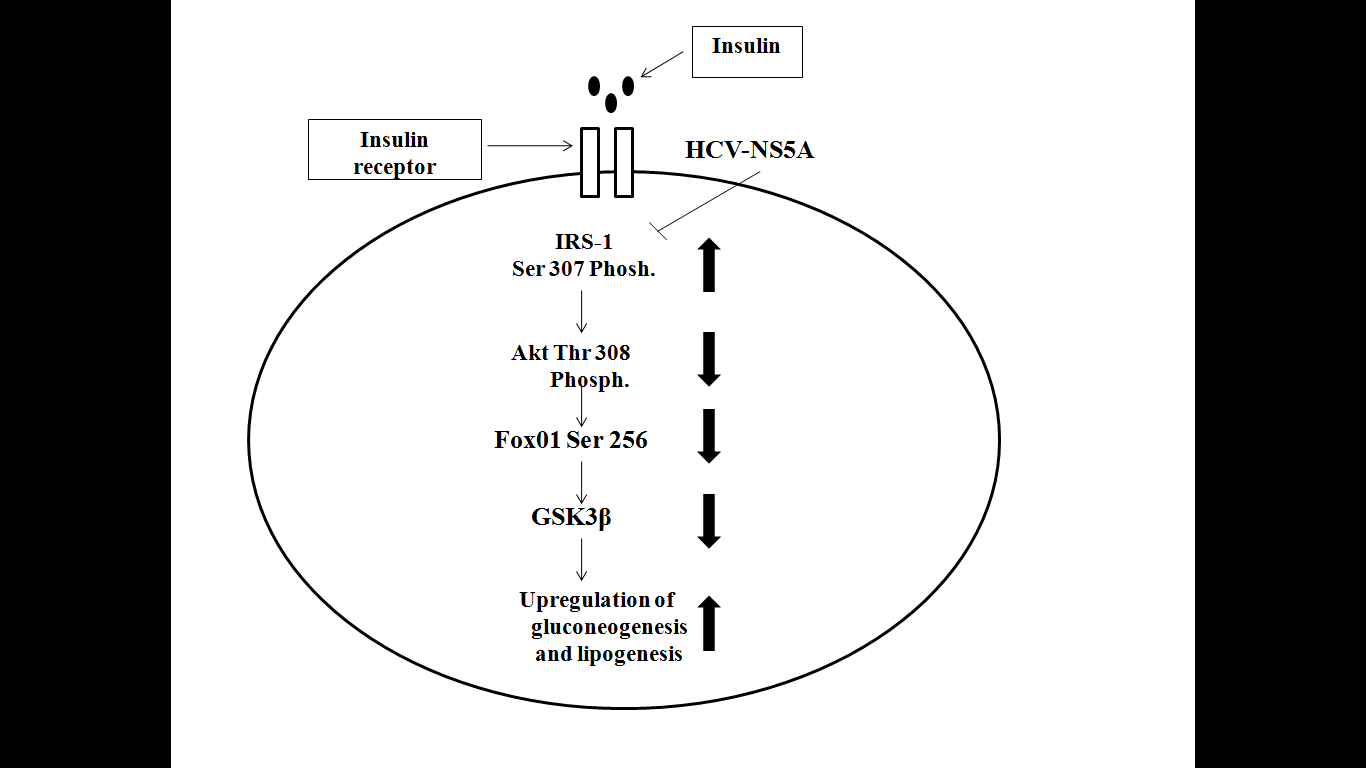
B)



a

a

**Figure 5** **Hepatitis C virus nonstructural protein 5A favors gluconeogenic gene expression.** Total cellular RNA was extracted from hepatitis C virus (HCV, A) nonstructural protein 5A (NS5A, B) transfected cells with vector control. The quantitative real-time polymerase chain reaction was performed for the targeted genes as described in materials and methods. 18S rRNA was used as housekeeping gene. Data represents mean of three independent experiments. a*P* < 0.05 *vs* control group. Data was analyzed by Graph Pad Prism. 2-tail Error bars represent SE of the data. TNF-α: Tumor necrosis factor-alpha; PEPCK: Phosphoenol pyruvate carboxykinase; G6P: Glucose-6-phosphatase; CREB: CRE-binding protein.



**Figure 6** **Schematic representation of hepatitis C virus nonstructural protein 5A induced insulin resistance.** Based on our findings, a model has been proposed that depicts various check points in insulin signaling pathway that gets modulated by nonstructural protein 5A (NS5A) protein. The up and down arrows represent upregulation and downregulation of proteins involved in insulin signaling cascades. The blunt headed line epresents the check point that gets blocked by NS5A as this protein favors Serine phosphorylation of insulin receptor substrate-1 (IRS-1) while under normal conditions tyrosine phosphorylation is known to take place. HCV: Hepatitis C virus.

**Table 1 Primers were used as described below**

|  |  |  |
| --- | --- | --- |
| **Gene** | **Sense primer** | **Anti-sense primer** |
| CREB | GATCTTAGTGCCCAGCAACC | GACGGACCTCTCTCTTTCGT |
| PEPCK | GGCTACAACTTCGGCAAATACC | GGAAGATCTTGGGCAGTTTGG |
| G6P | CATTGACACCACACCCTTTGC | CCCTGTACATGCTGGAGTTGAG |
| TNF-α | AGGCGCTCCCCAAGAAGACA | TCCTTGGCAAAACTGCACCT |

TNF-α: Tumor necrosis factor-alpha; PEPCK: Phosphoenol pyruvate carboxykinase; G6P: Glucose-6-phosphatase; CREB: CRE-binding protein.