Hepatitis C virus infection in vitro triggers endoplasmic reticulum stress and downregulates insulin receptor substrates 1 and 2 through upregulation of cytokine signaling suppressor 3

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Summary. – Hepatitis C virus (HCV) infection is highly prevalent worldwide and most of HCV infections enter into chronic phase subsequently leading to insulin resistance (IR) and clinical complications. Although the clinics of chronic HCV infection is well described, there is need to better understand the molecular mechanisms of HCV-induced IR. Therefore this study was aimed to unveil the role of host genes involved in the development of HCV-induced IR. For this purpose the expression of selected genes in HCV-infected and non-infected Huh-7 cells at various time post infection (p.i.) was assayed by real-time PCR. HCV infection was found to trigger endoplasmic reticulum (ER) stress response as demonstrated by an increase in the expression of calreticulin (Cal) gene but no change in the expression of Gadd153 gene. The infection also enhanced the expression of suppressor of cytokine signaling 3 (SOCS-3), responsible for the degradation of insulin receptor substrates (IRS). Moreover, it led to a decreased expression of key signaling molecules IRS-1 and IRS-2, unchanged expression of SOCS-7 and increased expression of downstream signaling molecule Akt. Altogether these findings indicate that the HCV infection induces ER stress and IR in Huh-7 cells in vitro.

Keywords: hepatitis C virus; insulin resistance; endoplasmatic reticulum stress; insulin receptor substrate; cytokine signaling suppressor

Introduction

HCV infection is a large scale pandemic that has proved to be a challenging threat in the past years because of its high morbidity and mortality. About 160 million people worldwide are reported to be infected with HCV (Lavanchy, 2011). Almost 85% of them develop chronic hepatitis C (CHC) that may lead to grave metabolic disorders including insulin resistance (IR), hepatic steatosis, hepatic fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC) (Hoofnagle, 2002). HCV has 6 major genotypes and many subtypes. Genotype 3a is the most prevalent in Pakistan (Idrees and Riazuddin, 2008). The only available treatment for HCV infection is ribavirin and interferon α (IFN-α) therapy. However the success rate of this treatment is no more than 50% (Negro, 2011) and currently no prophylactic vaccine is available.

Most of the pathological manifestations of CHC are because of the perturbation of the normal metabolic processes induced by interaction of HCV with the host cellular machinery. Several studies have shown that HCV is known to disturb glucose metabolism leading to IR. As a consequence type 2 diabetes mellitus (T2DM) may develop in the predisposed patients (Banerjee et al., 2008; Negro and Alaei, 2009). This results in presentation of all the typical symptoms of T2DM and deteriorates patient’s health condition due
to accelerated hepatic fibrogenesis, diminished response to IFN therapy and enhanced risk of HCC development. The molecular mechanism by which HCV induces IR needs to be further elucidated.

Insulin is an anabolic hormone that is secreted by the pancreatic β-cells and plays an essential role in cellular and extracellular homeostasis by regulation of glucose and lipid metabolism. Insulin performs its functions through a complex molecular cascade comprising of insulin receptors, IRS, phosphatidylinositol 3 phosphate kinase (PI3K), phosphoinositide dependent kinase 1 (PDK1), serine/threonine kinase (Akt) or protein kinase B (PKB) and forkhead-box (FOX) transcription factors. Akt and FOX downregulate the expression of phosphoenol pyruvate kinase (PEPCK) and glucose 6-phosphatase (G6P). This results in diminished gluconeogenesis and augmented glycogen synthesis (Wilcox, 2005).

HCV induced inflammation and subsequent upregulation of the SOCS proteins result in the proteosomal degradation of IRS. HCV proteins expression builds upfolded proteins burden in the ER and prompts the ER to activate the ER stress response characterized by enhanced expression of molecular chaperone genes like Cal and Gadd153. ER stress response adds to the severity of IR by activating protein kinase 2A (PP2A) that inhibits the activation of Akt (Bugianesi et al., 2012; Kaddai and Negro, 2011).

In this study, we investigated the role of selected host genes involved in the development of HCV-induced IR and ER stress. This was achieved by assaying the expression of selected genes in HCV-infected and non-infected Huh-7 cells by real-time PCR on various days p.i. We found out that the HCV infection in vitro led to (i) an increase in the expression of Cal, SOCS-3 and Akt genes, (ii) an unchanged expression of Gadd153 gene and (iii) a decreased expression of IRS-2 and IRS-3 genes. The present study identifies potential targets for development of new drugs which could be added to the standard anti-HCV regimen. Amelioration of patients’ health condition and increased incidence of sustained virological response (SVR) might represent the possible outcomes.

Materials and Methods

**Primer design.** Primers were designed in Primer3 Version 0.4.0 (http://frodo.wi.mit.edu/) for the real-time PCR of the selected genes. mRNA sequences of the selected markers, obtained from NCBI gene bank (http://www.ncbi.nlm.nih.gov/gene/), were used as templates for the primer design. Primer sequences designed by Primer3 were tested in Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) for specificity. The sequences of the used primers are provided in Table 1.

**Virus.** Sera samples positive for HCV were obtained from the ASAB diagnostic laboratory. Collected serum samples were subjected to infectivity titration and genotyping as described in our previous study (Tariq et al., 2013). The samples collected were of HCV genotype 3a with viral titer ranging from 1x10^6 to 5x10^6 IU/ml. The samples were tested to rule out co-infection with HBV and HIV.

**Cells.** Huh-7 cell line was used to establish in vitro infection of HCV. The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in humidified environment and 5% CO₂ in cell culture incubator. Cells were maintained in 60 mm tissue culture plates into 30–35% confluency, were washed with 1X PBS and then inoculated with HCV sera in 1:2 ratio. Infected cells were maintained overnight. 2 days p.i. HCV serum containing media was removed and adherent cells were washed thrice with 1X PBS. Washed cells were replenished with fresh media and incubation continued for 6 days. Negative control was done with normal human serum that was confirmed to be non-infected with HCV.

**RNA extraction, reverse transcription.** Cells 4 and 6 days p.i. were harvested and subjected to total RNA extraction using TRIzol reagent according to the manufacturer’s instructions. Major steps in total RNA extraction include cell lysis, phase separation, RNA precipitation, washing and solubilization. Quality and quantity of extracted RNA was assessed using spectrophotometer (Eppendorf BioPhotometer plus”). Quality of extracted RNA was also checked by loading 1μg RNA on 1% agarose gel and carrying out electrophoresis. Extracted RNA was treated with DNase I to eliminate any possible contamination of genomic DNA that could give false positive results. cDNA (1 μg) was synthesized by RevertAid™ Reverse Transcriptase (Thermo Scientific) using oligo(dT)₅ primers following the manufacturer’s instructions.

**Real-time PCR for HCV.** Cell culture medium from infected and non-infected cells was collected everyday and analyzed. Infection of the cells was confirmed by real-time PCR as described in our previous

### Table 1. Sequences of primers used for PCR amplification and quantification of target genes

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Product size (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH F</td>
<td>ACCACAGGTTGCACTATCCAC</td>
<td>453</td>
</tr>
<tr>
<td>GAPDH R</td>
<td>TCCACTCCCTTGTTGTGTA</td>
<td></td>
</tr>
<tr>
<td>IRS-1 F</td>
<td>GTACAGGCTCTCCATAGCG</td>
<td>184</td>
</tr>
<tr>
<td>IRS-1 R</td>
<td>GATCCTTCGGACACCTCTT</td>
<td></td>
</tr>
<tr>
<td>IRS-2 F</td>
<td>TGACTAAGGAGATGGCTT</td>
<td>249</td>
</tr>
<tr>
<td>IRS-2 R</td>
<td>GAGAAGGTCTGGGAACATTG</td>
<td></td>
</tr>
<tr>
<td>SOCS-3 F</td>
<td>CCACCTAGAAGCCTCTCGT</td>
<td>171</td>
</tr>
<tr>
<td>SOCS-3 R</td>
<td>CTCTCCGAGAGACGCTGAA</td>
<td></td>
</tr>
<tr>
<td>SOCS-7 F</td>
<td>CTCTCAATGCCCTCGTCTC</td>
<td>213</td>
</tr>
<tr>
<td>SOCS-7 R</td>
<td>CTGAGGGAGCGAGAAGAAAA</td>
<td></td>
</tr>
<tr>
<td>Calreticulin F</td>
<td>ACTACAGGAAAGCAAGACCGT</td>
<td>163</td>
</tr>
<tr>
<td>Calreticulin R</td>
<td>AGGGATCTCTCTCTGCTCTC</td>
<td></td>
</tr>
<tr>
<td>Gadd 153 F</td>
<td>CACTCTTGAGCTGCTCTC</td>
<td>176</td>
</tr>
<tr>
<td>Gadd 153 R</td>
<td>GGAGGATCCACTGCTCTTC</td>
<td></td>
</tr>
<tr>
<td>Akt F</td>
<td>CCCCTGAAACACCTCTCTG</td>
<td>209</td>
</tr>
<tr>
<td>Akt R</td>
<td>AGTCGCCATCTCCTCCTCTC</td>
<td></td>
</tr>
</tbody>
</table>
study (Tariq et al., 2013). Presence of HCV RNA in the infected cells but no signal in non-infected cells confirmed the infection.

**PCR for genes involved in IR.** PCR reaction conditions and thermal profiles were firstly optimized for all the primers for conventional PCR. The PCR reactions were carried out in PCR GeneAmp 9700 Thermocycler (Applied Biosystems). GAPDH, Cal, Gadd153, SOCS-3 and SOCS-7 were optimized at 2.5 mmol/l Mg\(^{2+}\) with the following reaction conditions: 95ºC for 5 min, 35 cycles of 95ºC for 30 sec, 57ºC for 40 sec, and 72ºC for 40 sec. For GAPDH and SOCS-7, annealing temperature of 59ºC was used. PCR for IRS-1 and IRS-2 were carried out at Mg\(^{2+}\) concentration of 1 mmol/l. An annealing temperature of 60ºC was used to run the PCR reaction while the remaining conditions and temperatures were same as described above. All PCR products were visualized on 2% agarose gel stained with ethidium bromide, along with 50 bp ladder on Dolphin Doc (S/N470883) gel documentation system (Wealtec).

**Real-time PCR for genes involved in IR.** PCR conditions and thermal profiles were also optimized for real-time PCR for all the markers. Quantification of all the markers under study was optimized using Maxima SYBR Green/ROX qPCR Mater Mix (Thermo Scientific) with 1 µg of synthesized cDNA as template. The PCR reactions were carried out in 7300 Real-Time PCR System (Applied Biosystems) with the following reaction conditions: 95ºC for 5 min, 40 cycles of 95ºC for 15 sec and 60ºC for 60 sec. A final dissociation stage was added with parameters: 95ºC for 15 sec, 60ºC for 20 sec and 95 ºC for 15 sec. Results were interpreted by analysis of amplification plots and Ct value calculation. Presence of secondary products or primer dimerization was ruled out by dissociation (melt) curve analysis. In order to have better evaluation, all PCR products were also visualized on 2% agarose gel stained with ethidium bromide, along with 50 bp ladder (Fermentas) on gel documentation system. RNA was extracted from infected and non-infected cells 4 and 6 days p.i. and 1 µg of cDNA was synthesized and then used as template to carry out the relative quantification of the expression of selected genes. PCR reactions for each marker from both infected and non-infected cells were set up in triplicate. Housekeeping gene GAPDH was used as a normalization factor to calculate a gene/GAPDH arbitrary unit (fold). At least 3 independent studies with replicates for each gene were used.

**Statistical analysis.** All statistical tests were carried out in GraphPad Prism 5.01. Hypotheses were tested by unpaired Student’s t-tests assuming unequal variances. Differences with P ≤0.05 were considered significant. Graphs were drawn in GraphPad Prism 5.01 with each column bar representing mean ± SEM.

### Results

**Expression of cellular genes involved in IR**

cDNA synthesized from cellular RNA was used as template for the amplification of selected genes involved in ER stress and IR. The PCR reactions were carried out as described before. In all reactions specifically amplified products of the selected genes were present (Fig. 1).

**HCV-induced expression of ER stress genes**

To evaluate the induction of ER stress by HCV, Cal and Gadd153 gene expression was measured in infected and non-infected cells by real-time PCR. Measurement of GAPDH mRNA expression was used as an internal control. The expression was studied at least in 3 separate experiments with similar results. PCR reactions for each marker from both infected and non-infected cells were set up in triplicate. A significant increase of 3.1-fold was observed in the gene expression of Cal in the infected cells as compared to the non-infected cells 4 days p.i. The level of Gadd153 gene expression in infected cells remained unaffected 4 days p.i. No significant change was observed in the expression levels of Cal and Gadd153 in infected cells 6 days p.i. (Fig. 2).

**HCV induced expression of SOCS genes**

To assess the expression profile of SOCS in case of HCV infection, SOCS-3 and SOCS-7 gene transcripts were quantified in infected and non-infected cells. A significant increase (3.3-fold) was observed in the gene expression of SOCS-3 in the infected cells 4 days p.i., while the level of SOCS-7 mRNA in infected cells as compared to the non-infected cells remained unaltered. The expression levels of SOCS-3 and SOCS-7 in infected cells were found to be significantly declined (0.3-fold) 6 days p.i. (Fig. 3).
AHMED, Q. L. et al.: INDUCTION OF IR AND ER STRESS BY HCV INFECTION IN VITRO

Fig. 2
HCV-induced expression of ER stress genes
Transcripts of Cal and Gadd153 genes were assayed in Huh-7 cells 4 days (a) and 6 days (b) p.i. by real-time RT-PCR. Asterisks indicate significant differences.

Fig. 3
HCV-induced expression of SOCS genes
Transcripts of SOCS-3 and SOCS-7 genes were assayed in Huh-7 cells 4 days (a) and 6 days (b) p.i. by real-time RT-PCR. Asterisks indicate significant differences.

Fig. 4
HCV-induced expression of IR genes
Transcripts of IRS-1, IRS-2 and Akt genes were assayed in Huh-7 cells 4 days (a) and 6 days (b) p.i. by real-time RT-PCR. Asterisks indicate significant differences.
HCV infection was studied by analyzing the a gene of the apoptotic stage of ER stress response (Xu et al., 2005). Overexpression of Gadd153 protein induces cell death through Bcl-2 inhibitable mechanism (Zinzsnzer et al., 1998). An interesting finding of this study is that no significant change in the transcriptional expression of Gadd153 was observed in the infected cells until 6 days p.i. The current study also revealed 2.6-fold significant increase in the transcript level of Akt in the infected cells 4 days p.i. Akt, which is first phosphorylated by mTOR-C2 and then by PDK1, exerts its role in insulin signaling by decreasing gluconeogenesis and increasing glucose transport into the cells and glycogen synthesis. Akt phosphorylation and downstream insulin signaling action are decreased due to IRS down regulation, impaired phosphorylation and degradation. HCV structural protein E2 has been shown to decrease the protein levels of IRS-1 and cause impaired insulin-induced Ser308 phosphorylation of Akt hence causing IR (Hsieh et al., 2012). It is suggested that an increase in the transcript level of Akt in response to HCV infection may be because of the cell survival and anti-apoptotic roles of Akt. Prevention of Gadd153 upregulation and Akt over-expression may be adaptation modification strategies of the HCV in order to avoid apoptosis and lead the cells towards carcinogenic pathway. ER stress and the subsequent unfolded protein response have been implicated in the onset of IR. Different studies revealed that severe ER stress induces harmful unfolded protein response that contributes to the development of IR and metabolic syndrome (Achard and Laybutt, 2012).

It is well established that HCV (Bode et al., 2003; Miyoshi et al., 2005) and many other viruses (Yokota et al., 2009) in which it has been demonstrated that human hepatocellular carcinoma cell line (HepG2) infected with HCV showed significantly elevated levels of SOCS-3 gene expression associated with reduced gene expression of IRS-1 and IRS-2. Hepatic SOCS-3 levels are significantly elevated in the patients with CHC who are non-responders to IFN therapy. Diminished SVR might be an outcome of induction of SOCS-3 proteins and the subsequent interaction with the IFN signaling pathway (Walsh et al., 2006).

Numerous studies have conclusively established that HCV infection results in an over expression of SOCS that degrade the IRS-1 and IRS-2 hence hindering the normal insulin signal transduction and cause IR (Kawaguchi et al., 2005).
In addition to decrease in the protein levels of IRS-1 and IRS-2 by proteosomal degradation, studies have also reported downregulation of mRNA expression of IRS-1 and IRS-2 in response to HCV infection.

The current study revealed that IRS-1 and IRS-2 expression significantly decreased to 0.36- and 0.43-fold respectively in the infected cells 6 days p.i. An extensive study involving 27 patients of non-alcoholic steatosis (NASH), 24 patients of non-alcoholic steatohepatitis (NASH), 71 patients infected with HCV and 29 patients with histologically normal liver, has revealed significant decline in the mRNA expression of IRS-1 and IRS-2 in the liver biopsies of the patients infected with HCV genotype 1 and 3 (Garcia-Monzon et al., 2011). HepG2 cells infected with HCV genotype 1b or 2 have showed considerable downregulation of IRS-1 expression as compared with cells treated with HCV negative serum (Persico et al., 2009). Downregulation in the expression of IRS-1 and IRS-2 impairs the downstream insulin signaling and leads to the development of IR.

On the basis of the present study findings, it may be inferred that HCV induces IR by the generation of ER stress and hyper expression of the SOCS which in turn downregulates the expression of key insulin signaling adaptor molecules IRS-1 and IRS-2. Hence downstream insulin signaling is impaired and IR may develop.

References


