Research Article

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The metabolic regulator PGC-1α links hepatitis C virus infection to hepatic insulin resistance

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Background & Aims: Chronic hepatitis C virus (HCV) infection is strongly associated with insulin resistance and diabetes mellitus. Peroxisome proliferator-activated receptor-gamma co-activator 1α (PGC-1α) is a transcriptional co-activator involved in the initiation of gluconeogenesis in the liver. Increased hepatic expression of PGC-1α has been implicated in insulin resistance. We investigated whether modulation of PGC-1α levels following HCV infection underlies HCV-associated hepatic insulin resistance.

Methods: HCV genomes were expressed in hepatoma cells followed by analysis of PGC-1α and gluconeogenesis levels.

Results: PGC-1α was robustly induced in HCV infected cells. PGC-1α induction was accompanied by an elevated expression of the gluconeogenic gene glucose-6 phosphatase (G6Pase) and increased glucose production. The induction of gluconeogenesis is HCV dependent, since interferon treatment abolishes PGC-1α and G6Pase elevation and decreases glucose output. Moreover, PGC-1α knockdown resulted in a significant reduction of G6Pase levels in HCV full length replicon cells, emphasizing the central role of PGC-1α in the exaggerated gluconeogenic response observed in HCV patients. Treatment of HCV replicon cells with the antioxidant N-acetylcysteine resulted in reduction of PGC-1α levels, suggesting that HCV-induced oxidative stress promoted PGC-1α upregulation. Finally, both PGC-1α and G6Pase RNA levels were significantly elevated in liver samples of HCV infected patients, highlighting the clinical relevance of these results.

Conclusions: PGC-1α is robustly induced following HCV infection, resulting in an upregulated gluconeogenic response, thereby linking HCV infection to hepatic insulin resistance. Our results suggest that PGC-1α is a potential molecular target for the treatment of HCV-associated insulin resistance.

Keywords: Hepatitis C virus; Peroxisome proliferator-activated receptor gamma co-activator 1α; Diabetes; Insulin resistance; Oxidative stress.

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Abbreviations: HCV, hepatitis C virus; PGC-1α, peroxisome proliferator-activated receptor gamma, co-activator 1α; G6Pase, glucose-6 phosphatase; RNA, ribonucleic acid; PI3, phosphatidylinositol 3-kinase; FLRP, full length replicon; SGR, subgenomic replicon; NS5A, non-structural protein 5A; SEAP, secreted alkaline phosphatase; NS, non-structural; SDS, sodium dodecyl sulfate; RIPA, radio immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; HPRT, hypoxanthine phosphoribosyltransferase; HA, hemagglutinin; ECL, enhanced chemiluminescence; IFN, interferon; SOCS, suppressors of cytokine signaling; FOXO1, forkhead box protein O1; ER, endoplasmic reticulum; JNK, c-Jun N terminal kinase; NAC, N-acetylcysteine; IRS-1, insulin receptor substrate-1.

Introduction

Chronic hepatitis C virus (HCV) infection is a major global health problem. Chronically infected patients may develop cirrhosis and liver cancer that are implicated in substantial morbidity and mortality [1]. Although the liver is the target organ for HCV infection, extrahepatic manifestations of HCV, including insulin resistance and diabetes mellitus, are frequently encountered in the clinical setting [2]. Insulin resistance was found to be a specific feature of chronic HCV associated with genotypes 1 and 4 and with high serum viral RNA levels [3]. Insulin resistance and diabetes mellitus also correlated with poor response to anti-HCV therapy, while eradication of the virus correlates with a marked improvement in insulin resistance [4].

The molecular mechanism by which HCV promotes insulin resistance is still undetermined. HCV infection was found to cause a postreceptor defect in the insulin signaling cascade [5]. In addition, the activation of proinflammatory mediators, such as nuclear factor-kappa-B and tumor necrosis factor-α, caused by the chronic infection state, interferes with insulin signaling and contributes to both hepatic and peripheral insulin resistance [6,7]. In agreement with the evidence suggesting a central role for reactive oxygen species in the development of insulin resistance [8], HCV infection is known to promote cellular oxidative stress through multiple mechanisms, including chronic inflammation, iron overload, and liver injury. Some of the HCV proteins were reported to directly contribute to this process [9,10].

Peroxisome proliferator-activated receptor gamma co-activator 1α (PGC-1α) is a transcription co-activator and a master regulator of gluconeogenesis that functions via interaction with transcription factors located on the promoters of gluconeogenic genes [11–13]. Although PGC-1α is scarcely detectable in the

liver under basal conditions, it is robustly induced in response to starvation, an effect that is mediated by the counter-regulatory hormonal response in which glucagon and glucocorticoids are involved [14,15]. PGC-1α has been shown to be overexpressed in livers of obese-hyperglycemic (*Ob/Ob*) mice [15], most probably reflecting the amplified hepatic gluconeogenesis that results in insulin resistance and diabetes mellitus. Interestingly, reducing hepatic oxidative stress in diabetic (*db/db*) mice resulted in a significant improvement in insulin resistance that was accompanied by a marked reduction of hepatic PGC-1α levels [16]. These data suggest that PGC-1α might be a central molecular player in the interplay between hepatic oxidative stress and insulin resistance. We hypothesized that the increased oxidative stress produced during HCV infection might lead to elevated liver PGC-1α expression, thereby promoting hepatic insulin resistance and diabetes mellitus.

Here, the role of PGC-1α in HCV-induced insulin resistance was investigated. By analyzing PGC-1α levels and the gluconeogenic response in HCV infected cells, as well as in HCV-infected patients, we show that PGC-1α has a central role in HCV-promoted exaggerated gluconeogenesis. These findings further clarify the molecular pathways leading to HCV induced insulin resistance and might have therapeutic implications.

Materials and methods

Cell culture, transfections, and treatments

HuH7 and 7.5 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Biological Industries, Israel). Non-essential amino acids (Biological Industries) were added to HuH7.5 cells. Human interferon-α (IFN) B2 was purchased from PBL (Piscataway, NJ), and N-acetyl-L-cysteine was purchased from Sigma.

Luciferase reporter assays

Cells were co-transfected with PGC-Luc reporter (a gift from B.M. Spiegelman (Dana-Farber, Harvard) [18]) and pM1-secreted alkaline phosphatase (SEAP, transfection efficiency control; Roche, Palo Alto, CA). Luciferase activity was determined 48 h post-transfection. SEAP activity was determined in the culture media using the Phospha-Light System (Applied Biosystems, Carlsbad, CA).

RNA analysis

RNA was extracted from cells by Tri-reagent (Sigma). After treatment with RNase-free DNase I (Promega), the RNA was subjected to reverse transcription using the Verso cDNA kit (Thermo, Waltham, MA). Real-time PCR was performed on the resulting cDNA to quantify the amounts of HCV, *PGC-1α*, and *G6Pase* mRNAs using the primers listed in Supplementary Table 1. Results were normalized to the mRNA levels of the housekeeping gene hypoxanthine phosphoribosyltransferase (*HPRT*) (Supplementary Table 1).

Protein analysis

Proteins were extracted from cells using radio immunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitors (Sigma) and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The following primary antibodies were used for Western blot: monoclonal mouse anti-HCV NS5A (Virostat, Portland, ME), mouse anti-PGC-1α antibody (Calbiochem, La Jolla, CA), mouse anti-tubulin antibody (Sigma) or mouse anti-actinin (Santa Cruz Biotechnology, Santa Cruz, CA). A goat anti-mouse antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a secondary antibody. Proteins were detected by chemiluminescence.

In vitro transcription and electroporation of HCV RNA

*Sca*I linearized plasmids encoding HCV genotype 1b (Bart79I, [17]) replicon were *in vitro* transcribed using Ribomax RNA production kit (Promega) under nuclease-free conditions. The plasmid encoding the full-length chimeric HCV J6/JFH1 genome was linearized with *Xba*I, followed by mung bean nuclease treatment. The linearized template was transcribed using a MEGAscript T7 kit (Ambion, Austin, TX). The DNA template was digested by DNase. RNA was extracted using TRI-Reagent. *In vitro* transcribed RNAs were electroporated into HuH7.5 cells, as described [19]. The pulsed cells were left to recover for 15 min and then plated in 6-well plates for further treatment.

PGC-1α knockdown

HIV-1-based lentiviral pGIPZ vectors containing shPGC-1α (clone V2LHS_71173) and scrambled shRNAs (clone RHS_4346) were purchased from Open Biosystems (Huntsville, AL). shRNAs harboring viruses were produced by transfection of 293T cells with 20 μg of shRNA plasmid together with the helper plasmids pCMVΔR8.2 (15 μg) and pVSVG (5 μg). Three days post-transfection, the culture medium was collected and filtered. HuH7 and FLRP cells in 6-well plates were incubated with 1 ml of infectious medium supplemented with polybrene (8 μg/ml; Sigma) for 8 h. The medium was replaced and cells were then incubated for additional 48 h.

Glucose production

The production of glucose was measured by an Amplex® Red Glucose/Glucose oxidase assay kit (Invitrogen). One million HuH7 or FLRP cells were seeded in a 6-well plate. The next morning, the culture medium was replaced with PBS (Biological Industries) supplemented with 20 mM sodium lactate. Following a 2-h incubation, media were collected and glucose levels were determined. The readings were normalized to cell viability determined by Alamar blue (Invitrogen). Gluconeogenesis levels were calculated by subtracting glucose levels in the presence of the gluconeogenic substrates from baseline glucose levels.

RNA extraction from formalin-fixed paraffin-embedded liver tissue

Paraffin-embedded liver tissues were taken from historical patients who were infected with HCV genotype 1, controls were HCV negative patients whom liver biopsies were taken for evaluation of elevated liver function tests from various other etiologies. The study was approved by the Tel-Aviv Sourasky Medical Center Ethics Committee (#0133-12-TLV), and was conducted in accordance with the principles of the Declaration of Helsinki. RNA extraction from formalin fixed paraffin embedded liver tissues was performed as described [20]. Briefly, tissue sections were deparaffinized using xylene and ethanol, air dried and incubated at 55 °C for 16 h in RNA lysis buffer (20 mM Tris pH 7.5, 20 mM EDTA, 1% SDS and 0.5 μg/μl proteinase K). RNA was extracted using Tri-Reagent (Sigma). Real-time PCR was preformed as described above.

Results

PGC-1α is elevated in HCV infected cells

We hypothesized that HCV replication induces PGC-1α. To verify this hypothesis, *PGC-1α* mRNA levels were first examined in the context of a newly established HCV infection, namely, electroporation of a genotype 1b subgenomic replicon (SGR, Bart 79I [17]). RNA was extracted 72 h following electroporation and analyzed for HCV and *PGC-1α* mRNA levels. HCV mRNA levels dramatically increased following electroporation (138-fold). A replicon with a lethal mutation in the RNA-dependent RNA polymerase (pol-) served as a negative control [17]. In agreement with our hypothesis, *PGC-1α* mRNA levels were significantly increased (7-fold) in these replicon cells (Fig. 1A). Since this replicon does not contain the viral structural genes (see scheme in Fig. 1), this results also indicates that the structural genes are not essential for PGC-1α elevation. Because our hypothesis links PGC-1α overexpression