Hepatitis C virus core protein genotype 3a increases SOCS-7 expression through PPAR-γ in Huh-7 cells

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Received 1 February 2010
Accepted 29 March 2010

Hepatitis C virus (HCV) core protein genotype 3a induces the expression of suppressor of cytokine signalling protein 7 (SOCS-7), which is partially involved in the development of insulin resistance. The aim of the present study was to investigate the mechanism through which the core protein regulates SOCS-7 expression. We have explored, in the in vitro model of Huh-7 cells expressing the HCV core protein of genotype 3a, whether the expression of SOCS-7 as well as of other members of the SOCS family (SOCS-1 and SOCS-3) was activated by the STAT3 pathway, using immunoblotting and real-time PCR upon alpha interferon (IFN-α) treatment. We found that, whilst IFN-α treatment induced STAT3 activation and consequently SOCS-1 and SOCS-3 upregulation in HCV genotype 3a core-expressing Huh-7 cells, SOCS-7 mRNA expression was independent of STAT3 and seemed to be modulated by peroxisome proliferator-activated receptor gamma (PPAR-γ) activity, as demonstrated by quantitative real-time PCR and immunoblot detection after treatment with the PPAR-γ agonist rosiglitazone or the PPAR-γ antagonist GW9262. In contrast to the other studied members of the SOCS family (1 and 3), which are regulated by STAT3 activation, SOCS-7 expression appears to be STAT3-independent and seems to be regulated instead by PPAR-γ. This is the first report proposing a molecular mechanism through which the HCV core protein (genotype 3a) modulates SOCS-7 expression.

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-strand RNA virus. Its genome encodes a polyprotein of >3000 aa that is cleaved post-translationally by host and viral proteases, yielding three structural (core, E1 and E2) and seven non-structural (p7 and NS2–NS5B) proteins. HCV is one of the leading causes of chronic liver disease worldwide and it is estimated that approximately 3% of the world population is infected (WHO, 2000).

Several studies have shown that HCV (Bode et al., 2003; Kawaguchi et al., 2004; Miyoshi et al., 2005) and other viruses (Yokota et al., 2004) are able to modulate the expression levels of the suppressor of cytokine signalling (SOCS) proteins, in order to inhibit cytokine signalling and finally to escape the immune response (Walsh et al., 2006; Huang et al., 2007). The SOCS family comprises eight proteins [CIS (cytokine-inducible SH2-containing protein) and SOCS-1 to -7] of structural and functional homology (Cooney, 2002). Over the past decade, SOCS proteins have been implicated in the regulation of over 30 cytokines, including interleukin (IL)-6, leukaemia-inhibitory factor (LIF), leptin, granulocyte colony-stimulating factor (G-CSF), IL-10, growth hormone and alpha, beta and gamma interferons (IFNs) (Cooney, 2002; Croker et al., 2008). In many instances, cytokine induction of SOCS protein expression has been shown to be dependent on the STAT signalling pathway (Auernhammer et al., 1999; Dickensheets et al., 2007). Furthermore, the induction of SOCS mRNA upon cytokine stimulation was inhibited when STAT activation by JAKs failed due to a mutation in the latter, indicating that SOCS genes are target genes of STATs and induced by the JAK/STAT pathway (Naka et al., 1997).

One of the common features of SOCS proteins is a 40 aa SOCS box motif, which is located at the C terminus of the protein and acts as an adaptor to facilitate the ubiquitination of target signalling proteins and their subsequent...
degradation by the proteasome system (Larsen & Röpke, 2002).

HCV infection, in addition to affecting the human immune response through SOCS protein modulation, is also linked intimately with the development of insulin resistance and metabolic syndrome (Sheikh et al., 2008).

In the present study, we intended to investigate whether the last member of the SOCS family, SOCS-7, which we recently demonstrated to be upregulated in human hepatoma Huh-7 cells expressing the HCV core protein of genotype 3a (Pazienza et al., 2009) and which is involved in the degradation of insulin receptor substrate 1 (IRS-1) (Pazienza et al., 2007) [one of the major players in the regulation of hepatic metabolism by insulin (Taniguchi et al., 2005)], is activated by the same JAK/STAT pathway. Moreover, as we and others have recently uncovered a role in HCV infection for peroxisome proliferator-activated receptor gamma (PPAR-γ) (de Gottardi et al., 2006; Pazienza et al., 2007; Dharancy et al., 2009), a member of the nuclear PPAR family and a promising therapeutic target for the inhibition of hepatic glucose production during metabolic syndrome (Edgerton et al., 2009), we tested the role of this nuclear receptor in HCV-induced SOCS-7 upregulation.

Here, we report that, upon IFN-α stimulation, SOCS-1 and SOCS-3 mRNA expression levels were increased significantly both in HCV core protein genotype 3a-transfected and in control cells, as expected; on the other hand, SOCS-7 levels were not stimulated by IFN-α. We found also that treatment with the PPAR-γ agonist rosiglitazone was able to reduce SOCS-7 expression and, conversely, the PPAR-γ antagonist GW9262 increased it in a dose-dependent manner, showing that PPAR-γ is involved in the regulation of SOCS-7 expression by HCV core protein genotype 3a.

RESULTS

SOCS-7 upregulation in Huh-7 cells expressing HCV core protein genotype 3a

We have demonstrated previously that SOCS-7 overexpression in hepatocytes expressing the core protein of HCV genotype 3a is involved in IRS-1 downregulation (Pazienza et al., 2007). As shown in Fig. 1, SOCS-7 expression is increased by about 2-fold at the protein level (Fig. 1b, c) in hepatocytes expressing the HCV core protein genotype 3a compared with control cells [HCV core 3a versus green fluorescent protein (GFP), \( P = 0.023 \)] or compared with untransfected cells [core 3a versus Huh-7, \( P = 0.031 \)]. Also, SOCS-7 mRNA expression levels were increased 48 h after transfection with HCV core protein genotype 3a (Fig. 2b), as described in our previous study (Pazienza et al., 2007). We have chosen 48 h as the time point for study because the maximum expression level of HCV core protein after transfection was reached (Fig. 1a), consistent with our previous findings (Abid et al., 2005; Pazienza et al., 2007, 2009).

SOCS-1, -3 and -7 expression in Huh-7 cells expressing HCV core protein genotype 3a

To better understand the kinetics of SOCS-1, -3 and -7 upon IFN-α treatment over time, we performed a time-course experiment incubating untransfected Huh-7 cells with IFN-α from 0 to 48 h (Fig. 2a). SOCS-1 mRNA expression reached

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**Fig. 1.** (a) Kinetics of HCV core expression by immunoblot detection of HCV core protein at different time points (0, 24 and 48 h) to establish the maximum level of expression. (b) SOCS-7 protein expression in Huh-7 cells expressing core protein genotype 3a or GFP, or in untransfected cells. Total protein (40 μg) extracted from Huh-7 cells expressing core protein genotype 3a or GFP, or from untransfected cells, was subjected to SDS-PAGE on a 10 % acrylamide gel, followed by immunoblot analysis with anti-SOCS-7, anti-β-actin or anti-core antibody. (c) Signal quantification by densitometry of SOCS-7 protein normalized to β-actin expression.
a peak after 8 h treatment; subsequently, it gradually decreased, but remained upregulated until 48 h. On the other hand, SOCS-3 mRNA expression was increased at 2 h and then remained upregulated until 48 h, whereas SOCS-7 mRNA was unchanged over time.

In an attempt to elucidate the possible molecular mechanism(s) through which the HCV core protein induces SOCS-7 expression, we examined whether the STAT3 signalling pathway was involved, as is the case for SOCS-1 and -3 (Naka et al., 1997). For this purpose, we activated the STAT3 protein by stimulation of hepatocytes expressing HCV core protein genotype 3a and control cells with IFN-α for 48 h and evaluated the SOCS-1, -3 and -7 mRNA expression levels. Whilst SOCS-1 and -3 mRNA levels were effectively increased upon IFN-α treatment both in untransfected hepatocytes (Fig. 2a) and in hepatocytes expressing HCV core or GFP (Fig. 2b), IFN-α failed to induce SOCS-7 expression under both conditions (Fig. 2a, b). On the contrary, SOCS-7 expression levels were reduced in cells expressing the HCV core protein upon treatment with IFN-α (3a versus 3a + IFN-α, \( P < 0.03 \); 3a + IFN-α versus GFP, \( P = \text{NS} \)) (Fig. 2b, c).

### STAT3 phosphorylation levels in Huh-7 cells expressing HCV core protein genotype 3a

We then examined the possibility that the increased level of SOCS-7 expression in the hepatocytes expressing HCV core protein genotype 3a and the lack of increase in SOCS-7 expression upon IFN-α treatment were due to a dysregulation of STAT3 activation. To find the optimal conditions for STAT3 phosphorylation, we incubated untransfected Huh-7 cells with 500 IU IFN-α ml\(^{-1}\) from 0 to 48 h. As shown in Fig. 3(a), IFN-α induced phosphorylation of STAT3 at 30 min and 2 h compared with baseline. After 48 h IFN-α treatment, we observed an increase in total STAT3 protein in both untransfected and transected cells (Fig. 3a–e) and a small but significant increase of STAT3 mRNA (Fig. 3c), which could explain the SOCS-1 and -3

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**Fig. 2.** (a) Time-course experiments of SOCS-1, -3 and -7 mRNA expression levels in control cells. The plot represents quantitative real-time PCR of cells treated at different time points with IFN-α at 500 IU ml\(^{-1}\). (b) SOCS-1, -3 and -7 mRNA expression before and after IFN-α stimulation at a concentration of 500 IU ml\(^{-1}\) for 48 h in HCV core protein genotype 3a or GFP-transfected Huh-7 cells. Total mRNA was extracted and analysed by quantitative real-time PCR for SOCS proteins. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \); ns, not significant. (c) SOCS-7 protein expression before and after treatment with IFN-α at 500 IU ml\(^{-1}\) for 48 h in Huh-7 cells expressing the HCV core protein or GFP, or in untransfected cells.
upregulation at 48 h. Next, we examined whether the STAT3 Tyr705 phosphorylation levels were dysregulated after treatment with IFN-α for 30 min in Huh-7 cells expressing the HCV core protein; no significant differences among the samples were observed (Fig. 3d). STAT3 activation upon IFN-α stimulation correlated with SOCS-1 and -3, but not SOCS-7, mRNA upregulation, suggesting that regulation of SOCS-7 expression is independent of STAT3 activation.

**SOCS-1, -3 and -7 expression after PPAR-γ modulation**

In our in vitro model of hepatocytes expressing the HCV core protein of genotypes 1b and 3a, we reported a genotype-dependent mechanism for the development of insulin resistance (Pazienza et al., 2007). In genotype 3a, SOCS-7 together with PPAR-γ have been implicated in the development of insulin resistance. As we did not observe any additive or synergistic effect on IRS-1 recovery after combined treatment with the PPAR-γ agonist rosiglitazone in concert with short interfering RNA-mediated silencing of SOCS-7 (Pazienza et al., 2007), we wondered whether, in genotype 3a, the increase in SOCS-7 expression was dependent on PPAR-γ modulation.

For this purpose, as a first step we confirmed the downregulated levels of PPAR-γ expression by immunoblot detection in hepatocytes expressing HCV core protein genotype 3a compared with control and GFP-transfected cells (3a versus GFP, P=0.028; 3a versus untransfected, P=0.037) (Fig. 4a, b). PPAR-γ mRNA levels were also found to be reduced in the presence of HCV core protein
genotype 3a (3a versus GFP, $P=0.046$) (Fig. 5a). We then proceeded to incubate Huh-7 cells with different concentrations of the PPAR-γ agonist rosiglitazone or the PPAR-γ antagonist GW9262. As shown in Fig. 4(c), whilst 48 h incubation with rosiglitazone reduced the expression of SOCS-7, the same-length incubation with GW9262 increased it, both in a dose-dependent manner. As for the expression of SOCS-1 and -3, no noticeable changes were observed after modulation of PPAR-γ activity with rosiglitazone and GW9262 (Fig. 4c).

**SOCS-7 modulation by PPAR-γ in Huh-7 cells expressing HCV core protein genotype 3a**

To assess whether regulation of SOCS-7 expression by PPAR-γ activity also occurs upon HCV genotype 3a core protein transfection, we incubated hepatocytes expressing HCV core protein genotype 3a with rosiglitazone (15 μM) or GW9262 (15 μM) for 48 h. Fig. 5(a) shows that SOCS-7 mRNA expression was also modulated by PPAR-γ in HCV core protein-expressing Huh-7 cells. Immunoblotting analysis confirmed that SOCS-7 protein levels followed the same trend (Fig. 5b, c). PPAR-γ mRNA levels were found to be slightly increased and reduced, respectively, upon rosiglitazone and GW9262 incubation in HCV core genotype 3a- and GFP-transfected cells (Fig. 5a). As controls for the modulation of PPAR-γ activity by rosiglitazone and GW9262, we examined whether these compounds (both used at 15 μM concentration) increased and reduced, respectively, the mRNA transcript levels of fatty acid synthase (FAS), a well-established target of PPAR-γ activity (Schadinger et al., 2005) (Fig. 5a). Noteworthily, variations in FAS mRNA levels upon HCV core genotype 3a infection and modulations of PPAR-γ activity by agonist and antagonist showed an inverse correlation with the changes in SOCS-7 mRNA levels under the same conditions (Fig. 5a). As the role of PPAR-γ was demonstrated indirectly through FAS mRNA measurement, and considering that PPAR-γ mRNA levels were found to be only slightly increased and reduced, respectively, upon rosiglitazone and GW9262 incubation in HCV core genotype 3a- and GFP-transfected cells (Fig. 5a), we conclude that efficient pharmacological modulation of PPAR-γ activity, involved in SOCS-7 regulation, may well also occur independently of variations in its expression levels.

**Fig. 4.** Immunoblot detection of PPAR-γ in Huh-7 cells expressing the core protein genotype 3a or GFP, or in untransfected cells (a). Forty-eight hours after transfection, cells were lysed and equal amounts of protein were loaded on a 10% polyacrylamide gel, separated by electrophoresis and immunoblotted with an anti-PPAR-γ antibody. (b) Signal quantification by densitometry of PPAR-γ protein normalized to β-actin expression. (c) Analysis of SOCS-1, -3 and -7 mRNA upon dose-dependent modulation of PPAR-γ activity by rosiglitazone (Ros) and GW9262 (GW). mRNA was extracted from untransfected Huh-7 cells treated with the indicated concentration of Ros or GW for 48 h and SOCS-1, -3 and -7 mRNA expression levels were assessed by quantitative real-time PCR.
DISCUSSION

The main finding of this study is the fine regulation of SOCS-7 protein by PPAR-γ activity. Thiazolidinedione derivatives, such as rosiglitazone, are insulin sensitizers known to bind and activate PPAR-γ, a nuclear receptor, which in turn regulates the expression of gene patterns beneficial for the cure of diabetes and metabolic disorders (Cho & Momose, 2008; Czaja, 2009). However, the precise mechanisms of action underlying the anti-diabetes effects of PPAR-γ agonists are poorly understood. Here, we provide new insights on the anti-diabetes actions of rosiglitazone based on its effects on SOCS-7 expression induced by HCV core protein genotype 3a. Using a pharmacological approach, we found that modulating PPAR-γ activity with a specific agonist (rosiglitazone) or with an antagonist (GW9262) leads to an inverse correlation between PPAR-γ activity and SOCS-7 expression in Huh-7 cells. Importantly, we have also demonstrated the ability of rosiglitazone to suppress SOCS-7 induction by the HCV core protein in a dose-dependent manner. Until now, only few studies have taken into consideration the link between PPAR-γ and SOCS proteins. It has been demonstrated that rosiglitazone rapidly induces the transcription of SOCS-1 and -3, which, in turn, inhibit JAK activity, reducing STAT3 phosphorylation in activated glial cells (Park et al., 2003), whilst in another work (Kanatani et al., 2007), it has been shown that the insulin sensitizer pioglitazone suppresses the expression of SOCS-3 and improves insulin sensitivity in in vivo and in vitro models of insulin resistance via PPAR-γ-dependent mechanisms. To our knowledge, this is the first report showing that pharmacological modulation of PPAR-γ activity can control SOCS-7 expression levels in hepatocytes.

Our findings show that, in the Huh-7 cell system, HCV core expression leads to a reduction in PPAR-γ expression that correlates with reduced levels of FAS mRNA. It remains to be established how HCV infection triggers hepatic steatosis without activating the PPAR-γ lipogenic pathway (Kim et al., 2007); in this respect, other PPAR isoforms, such as alpha (Tanaka et al., 2008), or other mechanisms, such as inhibition of microsomal triglyceride transfer protein (MTP) (Perlemuter et al., 2002; Mirandola et al., 2010), have been shown to play a role in the development of HCV-dependent hepatic steatosis. Whether the association between the core protein and the triglycerides is direct or indirect remains to be defined. It has been
shown that growing cells in delipidated medium reduce the triglyceride accumulation induced by HCV core protein genotype 3a, suggesting a lack of de novo synthesis of triglycerides (Abid et al., 2005) and suggesting that the core protein seems to promote a mere redistribution of fat-laden vesicles typically found in Huh-7 cells, rather than a true triglyceride accumulation. On the other hand, the core protein alone is able to modulate the expression of several cellular genes involved in lipid metabolism (Pazienza et al., 2009) and/or to promote de novo triglyceride synthesis (Lerat et al., 2009).

Interestingly, our data also indicate that, in contrast to SOCS-1 and -3, SOCS-7 is not upregulated by IFN-α. On the contrary, we found that IFN-α reduced SOCS-7 expression; we speculate that the reason for this downregulation could be due to the propensity of IFN-α to improve the insulin-resistance state and hepatic IRS-1/2 expression, as observed in biopsies of patients treated with IFN-α (Kawaguchi et al., 2007). Whether it is an indirect effect of IFN-α through the clearance of HCV or a direct effect through the modulation of the genes involved in hepatic IRS-1/2 expression that improves insulin resistance and beta-cell function remains to be elucidated.

PPAR-γ agonists have already been suggested as an adjuvant therapy in chronic hepatitis C (Elgouhari et al., 2008; Overbeck et al., 2008). In fact, there is the belief that correcting insulin resistance is a rational option in chronic hepatitis C patients (Negro, 2009), as it was observed that insulin resistance impairs the sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients (Romero-Gomez et al., 2005). However, new modalities of this correction have to be explored based on the mechanisms inducing insulin resistance, as insulin-sensitizing therapy should be tailored according to the infecting HCV genotype, as suggested by Negro (2009).

Although we have already shown that upregulated levels of SOCS-7 within hepatocytes expressing HCV core protein genotype 3a are involved in IRS-1 degradation, the novelty of this study resides in the identification of a PPAR-γ-driven mechanism to modulate SOCS-7 expression.

In conclusion, our results suggest that, in the in vitro model of Huh-7 cells expressing HCV core protein genotype 3a, the PPAR-γ agonist rosiglitazone may exert its insulin-sensitizing effect in part through the suppression of SOCS-7. Moreover, our findings identified SOCS-7 as a novel PPAR-γ molecular target in hepatocytes and indicate additional mechanisms for the therapeutic efficacy of PPAR-γ agonists.

**METHODS**

**Cell culture and transfection.** Human hepatoma Huh-7 cells were cultured at 37 °C in a 5 % CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U penicillin ml⁻¹ and 100 ng streptomycin ml⁻¹ (Invitrogen Life Technologies). pIRE2-EGFP plasmids containing the HCV 3a core-encoding region (Abid et al., 2005) were transfected into Huh-7 cells with Lipofectamine 2000 (Invitrogen Life Technologies) following the manufacturer’s instructions. Briefly, cells were incubated with Opti-MEM (Invitrogen Life Technologies) under standard conditions for the first 6 h after transfection and then the medium was changed to DMEM containing 10% FCS without antibiotics for another 42 h. For all experiments, we sorted GFP-expressing cells with a fluorescence-activated cell-sorting apparatus (FACS-Vantage; Becton Dickinson). For detection of STAT3 phosphorylation levels (Tyr705), we starved cells with serum-free DMEM before treatment with 500 IU IFN-α ml⁻¹ for 30 min.

**Antibodies and chemicals.** Rabbit polyclonal antibody directed against STAT3 and rabbit monoclonal phosphoSTAT3 (Tyr705) and PPAR-γ antibody were purchased from Cell Signaling Technology, Inc. SOCS-7 rabbit polyclonal antibodies and mouse monoclonal antibody against β-actin were obtained from Santa Cruz Biotechnology Inc. Mouse monoclonal antibody C7-50 against the core protein was from Alexis (Vinci-Bioclin). IFN-α was from Schering Plough Pharmaceuticals. Rosiglitazone and GW9262 were from Cayman Chemicals, kindly provided by Dr Ada Piepoli (Gastroenterology Unit, IRCCS ‘Casa Sollievo della Sofferenza’ Hospital).

**Quantitative real-time PCR.** Total RNA was extracted from Huh-7 cells transiently expressing HCV core protein genotype 3a or transfected with empty vector (pIRE2-EGFP) by using an RNeasy Mini kit (Qiagen) and subsequently digested by DNase I. cDNA was synthesized from 100 ng total RNA with SuperScript II RNase H(−) reverse transcriptase (Invitrogen) and random hexanucleotides. For real-time RT-PCR, we used the following SYBR Green QuantfiTect primers, all purchased from Qiagen: human SOCS-1 (QT00202475), human SOCS-3 (QT00244580), human SOCS-7 (QT00086608), human STAT3 (QT00068754), human PPAR-γ (QT00029841) and FAS (QT00014588). Reactions were set up in 96-well plates using a 7700 Real-Time PCR system (Applied Biosystems) and all samples were assayed in triplicate. Optical data obtained were analysed by using the default and variable parameters available in the SDS software package (version 1.9.1; Applied Biosystems). Expression levels of target genes were normalized by using the housekeeping control genes TATA-binding protein (TBP, QT00000721) and β-actin (ACTB1, QT00095431).

**Immunoblotting.** Huh-7 cells transfected with HCV core protein genotype 3a or with empty vector were lysed (Laemmli, 1970) in 2 x Laemmli sample buffer [250 mM Tris/HCl, pH 6.8; 500 mM diithiothreitol; 10% SDS; 0.5% bromophenol blue; 50 % (w/v) glycerol] supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics), 1 mM PMSF and 1 mM sodium orthovanadate. After boiling at 100 °C for 3 min, equal amounts of protein were loaded on 10% polyacrylamide gels and separated by electrophoresis. Protein transfer was performed on a PVDF membrane (Millipore). Membranes were blocked with 5% skimmed milk in wash buffer (20 mM Tris/HCl, pH 7.6; 140 mM NaCl; 0.1% Tween 20) and incubated with the different primary antibodies diluted appropriately in blocking solution. Following three washes, membranes were incubated with secondary horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (Bio-Rad) diluted 1:3000 in wash buffer. After three further washes, protein signals were revealed by chemiluminescence (ECL; Amersham Biosciences) and the signal was detected on an X-ray film (Amersham Biosciences). For quantitative measurement, films were scanned by densitometry and the spots corresponding to proteins were analysed by using the NIH image-analysis program Scion Image (Scion Corp.).
**Statistical analysis.** Results are expressed as means ± SEM. Comparisons were made using Student’s t-test. Differences were considered significant when P<0.05 (*), P<0.01 (**), or P<0.001 (***)

**ACKNOWLEDGEMENTS**

This work was supported by ‘Ministry of Italian Health’ grant R0902GA53 through the Research Unit of Gastroenterology, IRCCS ‘Casa Sollievo della Sofferenza’ Hospital, San Giovanni Rotondo (FG), Italy. Initial experiments performed by V.P. at Geneva University were funded by the Swiss National Science Foundation (grant no. 320000-116544). The authors declare that they have no competing interests.

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