Insulin-induced mTOR activity exhibits anti-hepatitis C virus activity

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Abstract. The mammalian target of rapamycin (mTOR) is one of the influential molecules for the anti-hepatitis C virus (HCV) action of interferon (IFN). IFN-induced mTOR activity, independent of phosphatidylinositol-3-kinase (PI3K) and Akt, is a critical factor for anti-HCV activity. mTOR activity is involved in signal transducers and activators of transcription (STAT)-1 phosphorylation and nuclear localization, and then double-stranded RNA-dependent protein kinase (PKR) is expressed in hepatocytes. Insulin (INS) is a major cytokine for metabolism and regulates the PI3K-Akt-mTOR signaling pathway in hepatocytes. Changes in mTOR activity have been reported in chronic HCV-infected patients with excess nutrition and INS resistance. Therefore, this experiment investigated whether INS increases anti-HCV activity via mTOR activity. This study used a genome-length HCV RNA (strain O of genotype 1b) replicon reporter system (OR6), derived from HuH7 cells. OR6 cells were pre-treated with rapamycin or LY294002 or siRNA, and the cells were treated with INS (0-300 nmol/l) or IFN (0-50 IU/ml) for 30 min to 48 h. The cells were lysed and analyses were carried out using the *Renilla* luciferase assay, western blotting or ELISA. INS induced the anti-HCV effects via mTOR activity, independently of STAT-1 tyrosine phosphorylation, in a dose- and time-dependent manner. INS-induced mTOR activation was found to be PI3K-Akt-dependent in OR6 cells. The combination of IFN and INS had an additive anti-HCV effect. The INS-induced mTOR activity was identified to be an anti-HCV signal independent of the STAT pathway in this study. mTOR activity may be associated with the HCV life cycle. Future studies should, therefore, attempt to identify new agents that activate mTOR to promote anti-HCV activity.

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Abbreviations: IFN, interferon; INS, insulin; HCV, hepatitis C virus; CHC, chronic hepatitis C; Jak, Janus kinase; STAT, signal transducers and activators of transcription; ISRE, IFN-stimulated regulatory element; PKR, double-stranded RNA-dependent protein kinase; Rapa, rapamycin; PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA

Key words: mammalian target of rapamycin, signal transducers and activators of transcription-1, interferon, hepatitis C virus, insulin

Introduction

At present, chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide (1). Although HCV is a hepatotropic virus, chronic HCV infection causes many metabolic disorders, including diabetes (2), insulin resistance (3) and hepatic steatosis (4). These metabolic disorders are related to an interferon (IFN) refractory condition (5-7). Treatment of HCV using a combination of pegylated IFN and ribavirin is effective in less than 50% of chronic hepatitis C (CHC) patients with genotype 1 virus (8). New anti-HCV agents, such as protease (9), polymerase (10) and cyclophilin inhibitors (11), have been developed to inhibit the HCV life cycle, but single application of these newly developed drugs is not effective enough for HCV eradication. Since IFN-α is the most basic agent for HCV treatment, it is necessary to improve the salvage rate of HCV infection by clarifying the efficacy of IFN treatment.

The most important intra-hepatocellular signal transduction of the IFN pathways is the Janus kinase (Jak)-signal transducers and activators of transcription (STAT) signal (12). Mammalian target of rapamycin (mTOR) is one of the influential molecules associated with IFN-induced anti-HCV action (13). The IFN-activated mTOR pathway plays an important regulatory role in the promotion of the IFN effect, including the anti-encephalomyocarditis virus effect (14). IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE)-dependent promoter gene activity. Human cytomegalovirus is inhibited by AMP-activated protein kinase-mediated inhibition of mTOR kinase (15). By contrast, vesicular stomatitis virus is mTOR-dependent (16). A relationship has been reported between the replication of hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70

S6 kinase pathway and regulates the replication of HCV (17). IFN-induced mTOR activity, independent of PI3K and Akt, is a critical factor for the anti-HCV activity, and Jak-independent mTOR activity involves STAT-1 phosphorylation and nuclear localization; subsequently, double-stranded RNA-dependent protein kinase (PKR) is expressed in hepatocytes (13).

mTOR activity has pleiotrophic functions, such as cell growth (18), nutrition control (19) and immunoregulation (20). However, there has been little examination of the influence that mTOR activity has on HCV proliferation. mTOR activity does not influence HCV-ISRE activity as the viral promoter has cap-independent translation (21). Although mTOR is a mRNA translational regulator that acts through phosphorylation of a downstream target such as 4E-BP and S6K (22), IFN-induced mTOR activity influences the phosphorylation of STAT-1 (13). Changes in mTOR activity in CHC patients with extra nutrition or with insulin resistance have been reported (23). However, the anti-HCV effect associated with mTOR activity remains to be sufficiently elucidated.

Insulin (INS) is a major cytokine for metabolism and acts via the PI3K-Akt-mTOR signaling pathway in hepatocytes (24). INS is indirectly related to HCV-associated INS resistance. Therefore, this study investigated whether INS increases the anti-HCV activity via mTOR activity.

Materials and methods

Reagents. INS was purchased from Eli Lilly Japan (Kobe, Japan). Recombinant human IFN-α2a was purchased from Nippon Rosche Co. (Tokyo, Japan). LY294002 and rapamycin (Rapa) were purchased from Calbiochem (La Jolla, CA, USA).

HCV replicon system. OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE (25), were used to examine the influence of the anti-HCV effect of IFN and INS. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin, and maintained in the presence of G418 (300 mg/l; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI, USA) and then subjected to a luciferase assay according to the manufacturer's protocol. The data were expressed as the relative luciferase activity.

Reporter gene assay. The OR6 cells were grown in 24-well plates. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers, IFN and INS, and the luciferase activities in the cells were determined using a luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative luciferase activity.

Cell viability assay. The cells were assessed using a colorimetric cell viability assay method. Cell viability was determined by a colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). Cell viability after 2 days of 100 IU/ml of IFN-α and 1000 nmol/l of Rapa treatment was expressed as a percentage of the viability in standard media without IFN-α and Rapa. Statistical significance was assessed using the Student's t-test, and a difference was considered to be statistically significant at $P<0.05$.

Western blotting and antibodies. Western blotting with anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-mTOR and anti-serine-2448 phosphorylated mTOR (Cell Signaling, Beverly, MA, USA) was performed as described later. OR6 cells were lysed by the addition of a lysis buffer (50 mmol/l Tris-HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l PMSF, 1 μg/ml each of aprotinin, leupeptin and pepstatin, 1 mmol/l sodium o-vanadate and 1 mmol/l NaF). The samples were separated by electrophoresis on 8-12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling), and the immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA).

mTOR kinase assay. The K-LISA mTOR Activity kit (Calbiochem) was used in this study. OR6 cells were incubated in the absence or presence of INS for 30 min with or without pre-treatment with Rapa for 12 h. The cells were washed two times with TBS and lysed by addition of a lysis buffer that was recommended by the manufacturer. The insoluble materials were removed by centrifugation at 10,000 rpm for 15 min at 4˚C, and the supernatants were collected and subjected to analysis of the mTOR kinase activity according to the manufacturer's protocol. The absorbance was measured with a Multiskan JX microplate reader.

siRNA transfection assay. Knockdown of the mTOR gene was performed using siRNA (Cell Signaling), and 100 nmol/l mTOR-specific and non-targeted siRNA as a control was used to transfect OR6 cells in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 10 IU/ml IFN or 300 nmol/l INS.

Results

INS exhibits anti-HCV activity with or without IFN. OR6 cells, a full-length HCV replication system, were used to examine the anti-viral effect of INS and IFN. The cells were incubated in medium that contained 0-100 nM of INS and 0-50 IU/ml of IFN for 48 h, and were harvested for a *Renilla* luciferase assay (Fig. 1). The relative *Renilla* luciferase activity decreased in an INS dose-dependent manner when the IFN concentration was 0 or 1 U/l. Cell viability in the presence of INS showed no statistically significant difference with or without IFN treatment for 24 or 48 h (data not shown). Rapa and LY294002 also showed no significant difference in comparison to the control (data not shown).

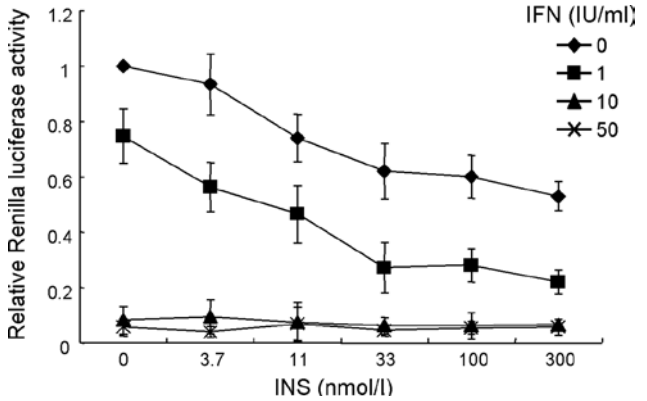

Figure 1. Effect of INS on HCV replication with or without IFN-α. OR6 cells were treated with 0-100 nmol/l INS and 0-50 IU/ml IFN. Forty-eight hours later, *Renilla* luciferase activity was determined by a luminometer (n=4). The data are expressed as the means ± SD and are a representative example of four similar experiments.

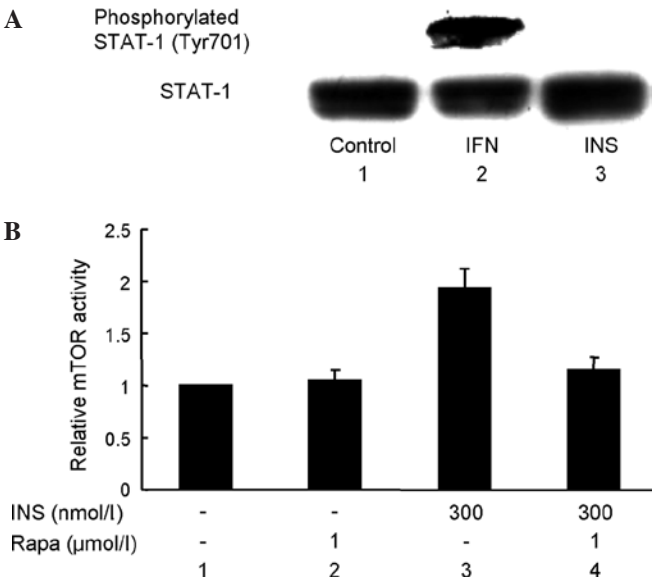

Figure 2. INS does not induce tyrosine phosphorylation of STAT-1 but induces mTOR kinase activity. (A) OR6 cells were incubated in medium only (control), and with IFN or INS for 30 min. Phosphorylation of STAT-1 at tyrosine-701 residue was analyzed by western blotting. (B) OR6 cells were not pre-treated (bars 1 and 3) or pre-treated with 1 μM Rapa (bars 2 and 4) for 12 h, and the cells were treated with 300 nM of INS (bars 3 and 4) for 30 min. Bars 1 and 2, cells not INS-treated. The mTOR kinase activity was determined by ELISA-based mTOR kinase activity assay kit (n=4). Data are expressed as the means ± SD. A statistically significant difference was observed between bar 3 vs. bars 1, 2 and 4 by the Student's t-test (P<0.05).

INS does not activate tyrosine on STAT-1, but induces mTOR kinase activity. OR6 cells were incubated in medium only or with 50 IU/ml of IFN or 300 nmol/l of INS for 30 min, and were lysed for western blotting (Fig. 2A). INS did not induce phosphorylation of Tyr 701 of STAT-1 (Fig. 2A, lane 3). IFN-induced anti-viral protein PKR was also not detected by western blotting under the same condition (data not shown). Treatment with 300 nmol/l of INS induced mTOR activity in OR6 cells. INS-induced mTOR kinase activity was suppressed in OR6 cells pre-treated with Rapa for 12 h before INS stimulation (Fig. 2B, bar 4).

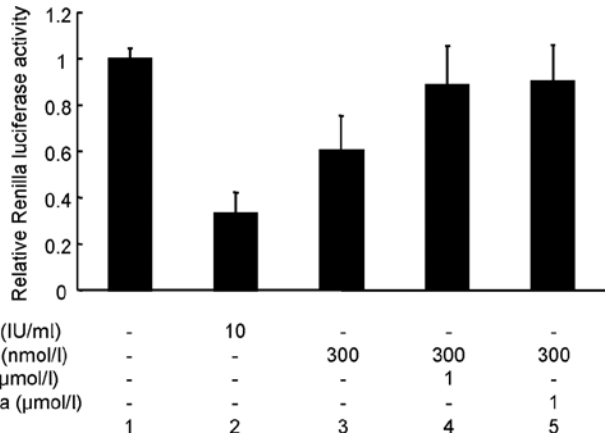

Figure 3. Changes in INS suppress HCV replication by PI3K and mTOR. OR6 cells were treated with 10 IU/ml IFN (bar 2) or with 300 nmol/l INS (bars 3-5) in the absence (bar 3) or presence of pre-treatment (bars 4 and 5) with LY294002 (LY) or rapamycin (Rapa) for 12 h. Bar 1, not treated. Forty-eight hours later, *Renilla* luciferase activity was determined by a luminometer (n=4). Data are expressed as the means ± SD and are a representative example of four similar experiments. A statistically significant difference was observed between bar 3 vs. bars 1, 4 and 5 by the Student's t-test (P<0.05).

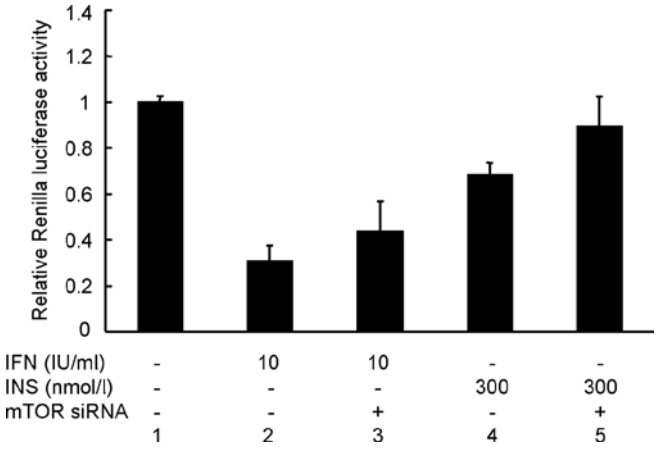

Figure 4. Changes in INS suppress HCV replication by siRNA against mTOR. OR6 cells were transfected by siRNA against mTOR (bars 3 and 5) or vehicle siRNA (bars 1, 2 and 4). One day later, the cells were treated with IFN (bars 2 and 3) or INS (bars 4 and 5). Thereafter, 1 day later, the *Renilla* luciferase activity was determined (n=4). Data are expressed as the means ± SD. A statistically significant difference was observed between bar 4 vs. bars 1 and 5 by the Student's t-test (P<0.05).

INS-induced anti-HCV activity is inhibited by blunted mTOR activity. The role of the PI3K-Akt-mTOR pathway in INS-induced anti-HCV activity was examined in OR6 cells. The cells were treated with INS after 12 h in the presence or absence of Rapa as an mTOR inhibitor, or LY294002 as a PI3K inhibitor (Fig. 3). Pre-treatment with LY294002 or Rapa attenuated the anti-HCV effect in comparison to INS alone. siRNA transfection for mTOR knockdown was used to explore the role of mTOR activity in INS-induced anti-HCV activity (Fig. 4). IFN-induced anti-HCV activity dependent on mTOR was inhibited by mTOR siRNA. Although the transfection efficiency of siRNA was barely 10%, INS-induced anti-HCV activity was clearly inhibited in mTOR siRNA-transfected cells (Fig. 4, bar 5).

Discussion

INS-induced mTOR activity exhibits anti-HCV action independently of STAT-1 phosphorylation. mTOR activated by INS was PI3K-Akt-dependent in hepatocytes. Notably, The STAT-1-independent anti-HCV activity did not induce the IFN-induced anti-viral protein PKR.

The mechanism of INS-induced anti-HCV activity is different from that of IFN. IFN-induced anti-HCV activity depends on IFN-induced anti-viral protein via Jak-STAT signaling (12). A genome-length HCV RNA (strain O of genotype 1b) replicon reporter system (OR6), which is an effective screening tool (25) has been used to identify a more effective therapy, especially for CHC patients with genotype 1. The OR6 system demonstrated the IFN-independent anti-HCV activity of statins (26). Another replicon system was used to demonstrate the IFN-independent anti-HCV activity of cyclosporine A (27). Both drugs have been associated with the life cycle of HCV and have an inhibitory effect on HCV replication (28,29). INS-induced anti-HCV activity may be associated with the life cycle of HCV, but not IFN-induced anti-virus protein. The susceptible point in the HCV life cycle is uncertain, but mTOR activity is an important factor which contributes to the inhibition of HCV proliferation.

Various relationships have been reported between mTOR activity and insulin resistance. mTOR was found to play a key role in IL-6-induced hepatic insulin resistance by regulating STAT3 activation and subsequent SOCS3 expression in an IL-6-induced insulin resistance model (30). Unsaturated fatty acids were found to inhibit PTEN expression in hepatocytes, leading to hepatic steatosis, insulin resistance, inflammation and cancer, by up-regulating microRNA-21 synthesis via an mTOR/NF-κB-dependent mechanism (31). mTOR activity was found to induce IRS serine phosphorylation leading to IRS degradation resulting in insulin resistance (23). HCV infection of hepatocytes stimulates insulin resistance through multifactors, including IRS degradation (32) and SOCS-3 expression (33). The present study of the association of mTOR activity and HCV proliferation suggests that insulin resistance is a condition that suppresses excessive proliferation of HCV and may be associated with chronic HCV infection.

INS-induced anti-HCV activity may be dependent on the PI3K-Akt-mTOR pathway, and one aspect of IFN-induced anti-HCV activity depends on mTOR activity (13). In this study, the combination of IFN and INS had an additive anti-HCV effect. Amino acids and INS have been demonstrated to have an additive effect on mTOR activity, since these effectors use different pathways to induce mTOR activity (34). Efficient anti-HCV activity via mTOR activity requires a combination of effectors that operate via different mTOR activation pathways. mTOR can be activated via three different pathways. The IRS-PI3K-Akt-mTOR pathway is stimulated by extracellular effectors, including INS and INS-like growth hormone (35). Activated mTOR inhibits the IRS function and generates negative feedback for IRS signaling (36). There is also nutrition-associated Akt-independent and wartmannin-dependent, or Akt-independent and adenosine monophosphate kinase-dependent mTOR activation. Amino acids and glucose inhibit Akt-dependent glycolysis through mTOR activation (37). Future studies should investigate agents that enhance IFN-induced Akt-independent mTOR activity via other mTOR-activated pathways.

INS-induced mTOR activity was found to be an anti-HCV signal-independent STAT pathway in this study. mTOR activity may be associated with the HCV life cycle. Future studies should therefore identify new anti-HCV agents that activate mTOR activity.

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