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Hepatitis C virus core protein transactivates the inducible nitric oxide synthase promoter via NF-κB activation

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Abstract

Intrahepatic levels of the inducible nitric oxide synthase (iNOS) are increased in chronic hepatitis C patients. As iNOS gene promoter contains Nuclear Factor (NF)-κB binding sites and hepatitis C virus (HCV) core protein activates NF-κB, the aim of this work was to study if HCV core protein transactivates iNOS promoter through NF-κB activation.

iNOS mRNA and protein were determined by RT-PCR and western blot in HepG2 cells. The effect of HCV core protein on iNOS promoter was assayed by cotransfecting HepG2 cells with the core protein expression plasmid pHCV-Co and p1iNOS-CAT or p2iNOS-CAT plasmids. Formation of NF-κB–DNA complexes was determined by electrophoretic mobility shift assay.

Transfection of HepG2 cells with pHCV-Co plasmid results in an increase in iNOS mRNA and protein levels. Cotransfection with pHCV-Co and p1iNOS-CAT or p2iNOS-CAT plasmids results in a transactivation of iNOS promoter, the presence of the proximal NF-κB binding site in the promoter being sufficient for the transactivation. Furthermore, the HCV core protein increases the formation of complexes between NF-κB and its binding sequence in the iNOS promoter. The expression of the NF-κB inhibitor IKB reverts the effect of the HCV core protein on the iNOS promoter.

In conclusion, HCV core protein transactivates iNOS gene promoter through NF-κB activation.

Keywords: HCV core protein; Nitric oxide; NF-κB

1. Introduction

Chronic hepatitis C virus (HCV) infection causes a wide spectrum of liver diseases ranging from mild chronic hepatitis to liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997; Saito et al., 1990). However, the mechanisms by which HCV causes liver damage are not fully defined although both the host immune response against infected hepatocytes as well as a direct cytopathic effect of viral gene products may be involved (Rehermann and Chisari, 2000; Gonzalez-Peralta et al., 1994). Regarding the pathogenesis of HCV infection, the inducible isoform of the nitric oxide synthase is up-regulated in the liver of patients with chronic hepatitis C (Mihm et al., 1997; Majano et al., 1998; Schweyer et al., 2000).

Nitric oxide synthases catalyze the conversion of L-arginine to L-citruline, a reaction that produces nitric oxide (NO) (Moncada et al., 1991). This reaction is catalyzed in hepatocytes by the inducible isoform of the nitric oxide synthase (iNOS) in response to endotoxins and proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interferon-γ (IFN-γ) (Nussler et al., 1992). The iNOS gene promoter contains multiple cytokine-responsive elements including several Nuclear Factor (NF)-κB binding sites (Nunokawa et al., 1994).

Although NO is involved in the non-specific immune response to infections (Karupiah et al., 1993; Kenneth, 1993; Nathan and Xie, 1994), its overproduction may have cytotoxic consequences (Lyons, 1995). In this regard, NO reacts with superoxide leading to the formation of peroxinitrite which provokes the nitration of tyrosine residues in cellular proteins (Stamler, 1994), and the level of intrahepatic nitrated proteins is increased in patients with chronic hepatitis C with respect to patients with non-viral liver disease, these levels being correlated with the histological severity of the hepatic damage (García-Monzón et al., 2000).

TNF-α, IL-1β, and IFN-γ are overexpressed during chronic liver inflammation (Andus et al., 1991; McCaughan et al., 1997) and up-regulated iNOS. However, the fact that hepatic iNOS expression is positively correlated with the intrahepatic HCV-RNA levels (Mihm et al., 1997) suggests

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that an HCV gene product may directly activate iNOS expression.

HCV core protein, apart from its structural functions (Santolini et al., 1994; Yasui et al., 1998), is a transcriptional regulator of cellular and viral promoters (Ray et al., 1995) and activates several intracellular signaling pathways (Chen et al., 1997; Tsuchihara et al., 1999; Kato et al., 2000) including that of NF-κB (Zhu et al., 1998; Shrivastava et al., 1998; You et al., 1999; Tai et al., 2000). Since the iNOS promoter contains functional NF-κB binding sites and that NF-κB binding to its sequence is required for the induction of the iNOS expression (Xie et al., 1994), it might be speculated that the HCV core protein may directly activate iNOS transcription in infected hepatocytes.

To test this hypothesis we determined if HCV core protein is able to activate iNOS expression at the transcriptional level and if this activation is mediated by NF-κB.

2. Materials and methods

The coding region of the HCV core protein was amplified by reverse transcription–polymerase chain reaction (RT-PCR) using the total RNA isolated from 250 μl of an HCV-RNA positive serum sample. The cDNA synthesis and the first PCR round were performed using the Superscript One-Step™ RT-PCR System (Gibco BRL) and the primers CSE (5′AGGTCTCGTAGACCGTGCAC3′) and CAE (5′TATATCCCGGACACGTTGTGC3′). The reaction was performed at 48 °C for 45 min followed by 2 min at 94 °C and by 30 cycles at 94 °C 1 min, 54 °C 1 min, 68 °C 1 min. The second PCR was performed using 5 μl of the first PCR product and the primers CS1 (5′GCTAGCATGAGCACGAATCCTAAACC3′ including the Nhe I target sequence at its 5′ end) and CA1 (5′GCGGCCGCTTAAGCGGAAGCTGGGATG3′ containing the Not I target sequence at its 5′ end). The conditions of the second PCR were those of the first PCR except that the annealing temperature was 59 °C. The 592 bp PCR product was cloned using the pCRII TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced in an ALF™ Express DNA sequencer (Pharmacia Biotech AB, Uppsala, Sweden). Then, cDNA was excised by Not I/Nhe I digestion and subcloned in the pCI plasmid (Promega Co., Madison, WI) to generate pHCV-Co plasmid.

The p1iNOS-CAT plasmid, in which the chloramphenicol acetyl transferase (CAT) gene is under control of the 1.1-kb fragment of the hepatic iNOS promoter, p2iNOS-CAT plasmid, containing the proximal 260 bp of the iNOS promoter and pRSV-IκB plasmid, expressing the NF-κB inhibitor IκB, have been described (Amaro et al., 1999). The pNF-κB-Luc plasmid (Stratagene, La Jolla, CA) contains luciferase gene controlled by a minimal promoter fused to 5 tandem copies of the NF-κB binding sequence.

2.1. Cell line and transfections

The human hepatoblastoma cell line HepG2 was used in this study. Cells were grown in DMEM (Imperial, Andover, UK) supplemented with 10% heat-inactivated fetal bovine serum (Imperial) and antibiotics (100 U/ml Penicillin and 100 μg/ml Streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2.

HepG2 cells (10^6 cells) were transfected with 3 μg of the pHCV-Co plasmid using the Lipofectamine™ Plus Reagent (Gibco BRL). To test whether the HCV core protein activates the NF-κB signaling pathway in our system, HepG2 cells were transfected with 2 μg of pNF-κB-Luc and 3 μg of pHCV-Co or pCI plasmids. To determine if the HCV core protein transactivates the iNOS promoter, cotransfection assays were performed with 2 μg of the p1iNOS-CAT or the p2iNOS-CAT plasmids, and increasing amounts of pHCV-Co (1, 3, and 5 μg). To confirm the implication of NF-κB in the transactivation of the iNOS promoter by the HCV core protein, HepG2 cells were transfected with 2 or 4 μg of the pRSV-IκB plasmid and p1iNOS-CAT and pHCV-Co plasmids. To keep constant the DNA concentration, the plasmid pGEM 3Z (Promega) was used as filler.

2.2. Analysis of human hepatocyte iNOS mRNA by reverse transcription–polymerase chain reaction

RNA from transfected cells was isolated using the Trizol LS Reagent (Gibco BRL). After ethanol precipitation, RNA was digested with RNase free-DNase (Promega) for 30 min at 37 °C. After phenol extraction and ethanol precipitation, RNA concentration was determined and 1 μg of RNA was used for amplification of iNOS and β-actin mRNAs by RT-PCR in the presence of ^{32}P-ATP as described by Amaro et al. (1999).

The PCR products were resolved by 8% polyacrylamide gel electrophoresis followed by autoradiography of the dried gel. Quantitation of the amplified products was performed by densitometric analysis using a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

2.3. Western blot detection of HCV core and iNOS proteins

HepG2 cells transfected with pHCV-Co plasmid were rinsed in 1× PBS and lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonylfluoride, 10 μmol/l leupeptine, 1 mmol/l benzamidine, and 100 μM Na_3VO_4) for 60 min on ice and then centrifuged at 10,000 × *g* for 10 min at 4 °C. Protein concentration was determined by the BCA assay (Pierce, Rockford, IL). After that, 10 μg of total proteins were boiled for 5 min in SDS–PAGE sample buffer and electrophoresed in 8% polyacrylamide gels. Then, the gel was blotted to a Dextran PVDF membrane (Schleicher & Schuell, Dassel, Germany), and after blocking in

10% non-fat milk, membrane was incubated for 1 h with a mouse-specific monoclonal antibody against the HCV core protein (Monotope TM antibody to HCV, ViroStat, Portland, ME) at 1/50 dilution, with an 1/1000 dilution of an anti-iNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) or with an 1/500 dilution of an anti-actin antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After washing, membranes were incubated with a 1/10,000 dilution of the corresponding peroxidase-conjugated antibody (Dakoppatts, Lostrup, Denmark) for 30 min at room temperature. Finally the blots were revealed with the enhanced chemiluminescent substrate (Supersignal West Pico Chemiluminiscent Substrate, Pierce).

2.4. Chloramphenicol acetyl transferase assay

Forty-eight hours after transfection, cell lysates were harvested and CAT analysis was performed using the CAT Enzyme Assay System (Promega). Equal amounts of protein were used and adjusted to ensure that enzyme activity remained within the linear (<50% conversion) range. Quantitation was performed by densitometric analysis of spots on thin-layer chromatography plates. Transfections were repeated at least four times to verify the reproducibility of the results. The results of CAT analysis are presented as the mean ± S.D. of the fold induction obtained in all independent experiments.

2.5. Luciferase assay

Luciferase activity was determined using the Dual Luciferase Kit Assay System (Promega). Bioluminiscence was measured in a 1450 Microbeta TriLux luminometer (Perkin-Elmer Wallac, Turku, Finland).

2.6. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were obtained from untransfected and pHCV-Co transfected HepG2 cells washed in 1× PBS and lysed in buffer A (10 mM Hepes-KOH, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) at 4 °C for 10 min. After centrifugation at 12,000 × *g* for 10 s, the pellets were extracted with 20 μl of buffer C (20 mM Hepes-KOH, pH 7.4, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 25% glycerol) at 4 °C for 20 min. Samples were cleared by centrifugation at 4000 × *g* for 2 min at 4 °C, and the protein concentration was measured by BCA assay. Samples were stored at −70 °C until use.

A double-stranded oligonucleotide containing the NF-κB binding site (5′-AGCTGGGGACACTCCCTTTGG-3′) of the hepatic iNOS promoter located at −102 base pairs from the transcriptional start site was labelled with ^{32}P-γATP by the T4 polynucleotide kinase (Promega) at 37 °C for 1 h. The radiolabeled probe was purified with a MicroSpin G-25 column (Amersham Pharmacia).

In the binding reaction, 50,000 cpm of the probe were incubated with 5 μg of nuclear extracts at 4 °C in a buffer containing 20 mM Tris (pH 7.5), 75 mM KCl, 1 mM DTT, 12% glycerol, 1 μg/μl poly(dI-dC), and 5 mg/ml BSA for 30 min at room temperature. Protein–DNA complexes were separated by electrophoresis on MDE acrylamide gels (FMC Corporation, Rockland, ME). Gels were dried and exposed to PDS film (Kodak, France).

As specificity control, the mutant oligonucleotide 5′-AGCTG**AAA**ACAC**AAA**CTTTGG-3′ was also used. Furthermore, a 50-fold molar excess of the cold probe containing the iNOS NF-κB binding site or the mutant oligonucleotides was used as homologous and heterologous competitors.

3. Results

3.1. Expression of HCV core protein and activation of NF-κB in transfected cells

To test whether the pHCV-Co plasmid drives the expression of the HCV core protein, cellular extracts from transfected HepG2 cells were analyzed by western blot. As shown

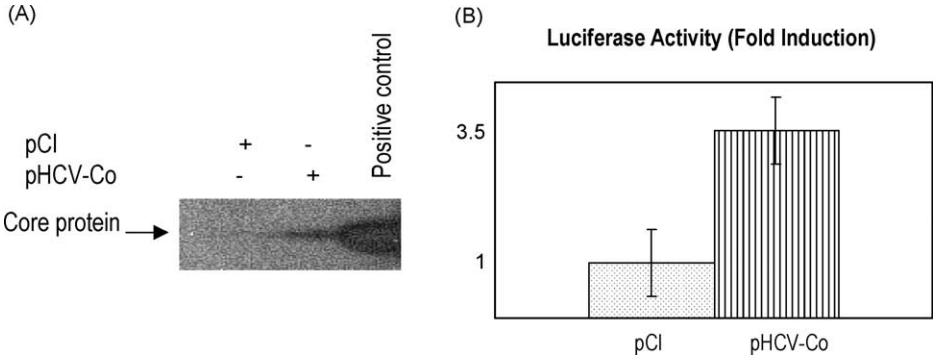

Fig. 1. (A) Western blot analysis of HCV core protein expression in HepG2 cells transfected with the plasmids pCI (Promega) or pHCV-Co plasmids. Positive control: recombinant HCV core protein (Chemicon Inc., Temecula, CA). (B) Relative luciferase activity in HepG2 cells cotransfected with the plasmid pNF-κB-Luc and the pCI or pHCV-Co plasmids.

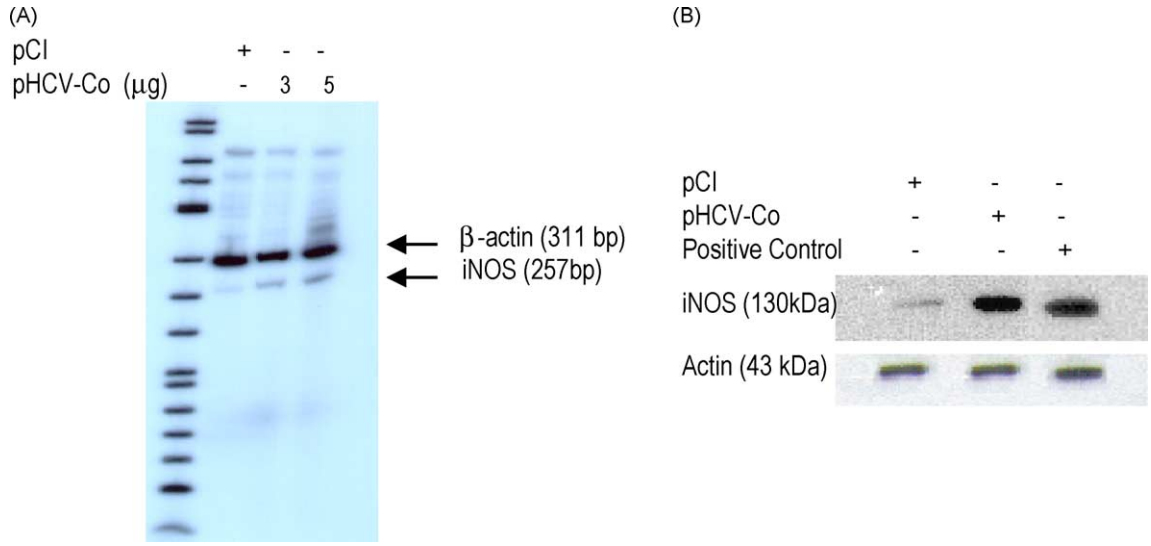

Fig. 2. (A) RT-PCR analysis of iNOS and β-actin mRNA levels in HepG2 cells transfected with increasing amounts (3 and 5 μg) of pHCV-Co plasmid. As shown in the figure, an increase in iNOS mRNA levels was found in HCV core protein expressing cells. (B) iNOS and actin protein levels in HepG2 cells transfected with the pHCV-Co plasmid.

in Fig. 1A, a protein with an apparent molecular weight of 19 kDa that specifically reacted with the anti-HCV core protein monoclonal antibody was detected in transfected cells but not in non-transfected or in cells transfected with the pCI vector.

To confirm the functionality of the HCV core protein in activating the NF-κB signaling pathway in our experimental conditions, HepG2 cells were cotransfected with the pNF-κB-Luc plasmid and the pHCV-Co or pCI plasmids. The expression of the HCV core protein induced a 3.5 ± 0.57-fold increase in the luciferase activity with respect to that in cells transfected with the pCI plasmid (Fig. 1B).

3.2. Activation of iNOS transcription by HCV core protein

To determine if HCV core protein induced an increase in the mRNA levels of the hepatic iNOS, total RNA isolated from HepG2 cells transfected with pCI or pHCV-Co plasmids was used to coamplify the iNOS and the β-actin (internal control) mRNAs by RT-PCR. As shown in Fig. 2A, the expression of HCV core protein increased iNOS mRNA levels in a dose-dependent manner, while the β-actin mRNA levels remained unaffected.

Similarly, the levels of iNOS protein were also increased in cells expressing the core protein with respect to that in HepG2 cells transfected with the empty pCI vector (Fig. 2B).

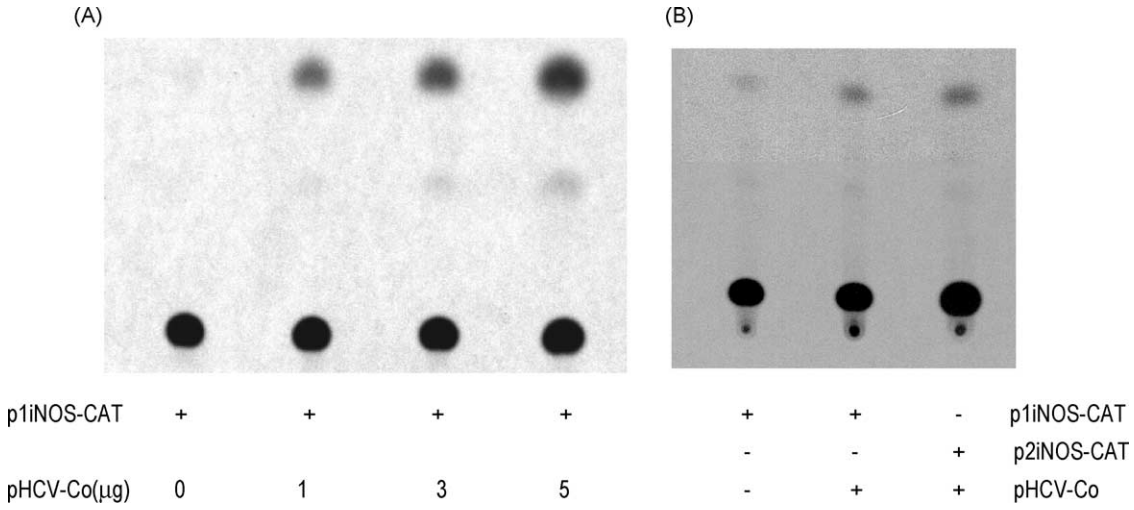

Fig. 3. (A) CAT activity in HepG2 cells cotransfected with the p1iNOS-CAT plasmid and increasing amounts of the pHCV-Co plasmid (1, 3, and 5 μg). (B) CAT activity in HepG2 cells cotransfected with the pHCV-Co plasmid and the p1iNOS-CAT or p2iNOS-CAT plasmids, showing that the level of HCV core protein mediated transactivation of both iNOS promoter fragments was similar.

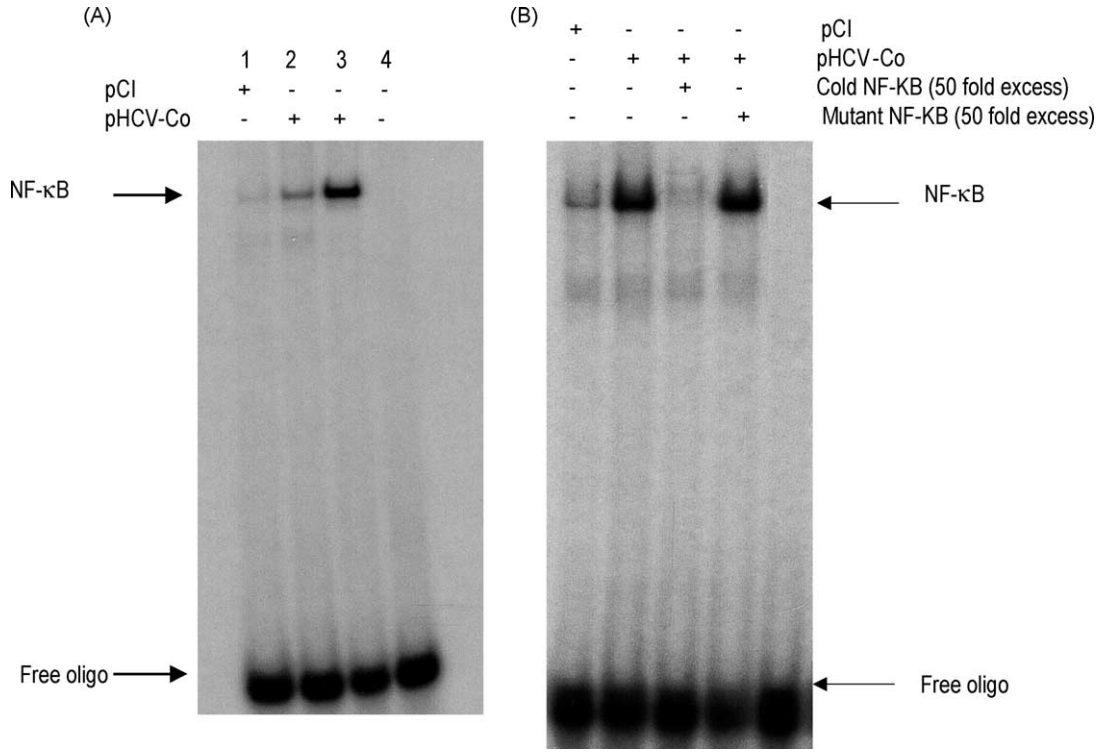

Fig. 4. (A) NF-κB mobility shift assay in nuclear extracts from HepG2 cells transfected with increasing amounts of the plasmid pHCV-Co. (B) EMSA specificity controls, including excess of the non-radioactive oligo probe and with a mutant probe.

3.3. Activation of the iNOS promoter by the HCV core protein

To investigate if the increase in the iNOS mRNA levels induced by the HCV core protein was due to the transcriptional activation of the iNOS gene promoter by the viral protein, HepG2 cells were cotransfected with the p1iNOS-CAT plasmid and increasing amounts of the pHCV-Co plasmid. The CAT analysis showed that the HCV core protein up-regulates iNOS gene promoter activity in a dose-dependent manner (Fig. 3). Cotransfection with the p2iNOS-CAT and the pHCV-Co plasmids, showed that HCV core protein was able to transactivate this minimal iNOS promoter with an activity similar to the 1.1 kb iNOS promoter plasmid, suggesting that the presence of the proximal NF-κB binding site of the iNOS promoter was sufficient for the transactivation mediated by the HCV core protein.

To test whether the expression of HCV core protein induces the formation of complexes between NF-κB and the oligonucleotide containing the NF-κB binding site of the iNOS promoter, EMSA were performed using nuclear extracts from pHCV-Co transfected and untransfected HepG2

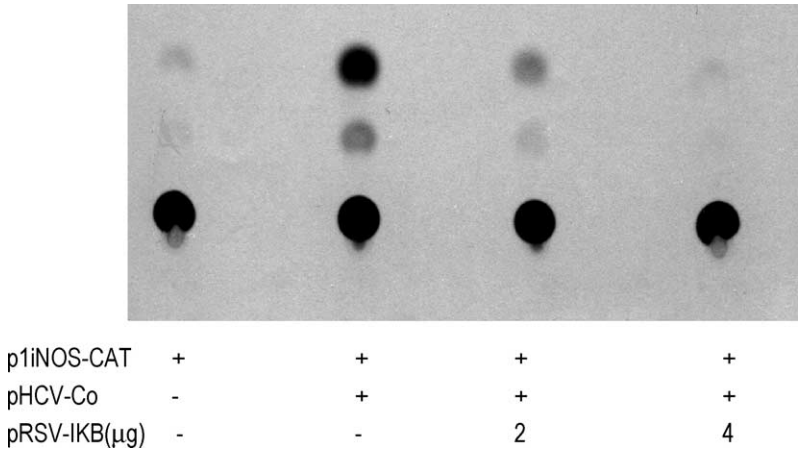

Fig. 5. CAT analysis of HepG2 cells transfected with p1iNOS-CAT, pHCV-Co, and increasing amounts of the plasmid expressing the NF-κB inhibitor IκB (pRSV-IκB).

cells. By these assays, it was observed that the level of the NF-κB–DNA complexes was higher in pHCV-Co transfected than in untransfected HepG2 cells (Fig. 4A). The specificity of the assay was demonstrated by the lack of complexes when the mutated oligonucleotide was used in EMSA and by the decrease in the level of these complexes when an excess of cold homologous oligonucleotide was added to the binding reaction but not when the mutated oligonucleotide was used as competitor (Fig. 4B).

Finally, to determine if the up-regulation of the iNOS gene promoter by the HCV core protein is mediated by the activation of NF-κB, HepG2 cells were cotransfected with p1iNOS-CAT and pHCV-Co plasmids and increasing amounts of the IκB expression plasmid pRSV-IκB. As shown in Fig. 5, expression of IκB inhibits HCV core protein transactivation of the iNOS promoter in a dose-dependent manner.

4. Discussion

Nitric oxide is a free radical gas molecule that possesses hepatoprotective and antiviral activities (Moncada et al., 1991). However, its long-term overproduction may be toxic for the hepatocytes (Lyons, 1995). Previous works have described that iNOS, the enzyme responsible for NO generation in hepatocytes (Nussler et al., 1992), is overexpressed in the liver of patients with chronic HCV infection (Mihm et al., 1997; Majano et al., 1998; Schweyer et al., 2000).

During liver inflammation, iNOS transcription is up-regulated by proinflammatory cytokines such as TNF-α, IL-1β, and IFN-γ (Nussler et al., 1992). However, certain viral proteins such as the hepatitis B virus encoded X protein may also directly activate iNOS transcription (Amaro et al., 1999; Majano et al., 2001). This finding, together with the fact that iNOS expression is positively correlated with intrahepatic HCV-RNA levels in chronic hepatitis C patients (Mihm et al., 1997), leads to the hypothesis that an HCV protein could be implicated in the activation of the hepatic iNOS transcription in patients chronically infected by this virus. As the HCV encoded core protein is able to modulate several intracellular signaling pathways (Chen et al., 1997; Tsuchihara et al., 1999; Kato et al., 2000; Zhu et al., 1998; Shrivastava et al., 1998; You et al., 1999; Tai et al., 2000), the present work has been performed to study whether this HCV product can activate hepatic iNOS transcription.

By RT-PCR using total RNA isolated from HepG2 cells transfected with different amounts of the plasmid encoding the HCV core protein, we have shown that iNOS mRNA levels increased in a dose-dependent manner and that this increase in the iNOS mRNA was accompanied by a rise in the intracellular levels of the iNOS protein, indicating that the HCV core protein may be one of the viral products responsible for the iNOS increase that has been documented in patients with chronic HCV infection.

As iNOS mRNA may be regulated at both transcriptional and post-transcriptional levels under certain circumstances (Amin et al., 1997; Zhao et al., 1997), we tested next if the HCV core protein was able to up-regulate the iNOS promoter activity. By cotransfecting HepG2 cells with the p1iNOS-CAT and pHCV-Co plasmids, CAT activity increased in parallel with the amount of the core expression plasmid cotransfected. This finding indicates that the HCV core protein transactivates the iNOS promoter. Furthermore, HCV core protein was also able to transactivate a 260-bp fragment of the iNOS promoter containing the proximal NF-κB binding site, suggesting that the transactivation of the iNOS promoter by the HCV core protein is mediated by the activation of the NF-κB signaling pathway.

Similar results of NF-κB activation were found by EMSA. In these experiments, an increase in the binding of NF-κB to its binding sequence in the iNOS promoter was observed in nuclear extracts from pHCV-Co-transfected cells when comparing the shift bands with those obtained using nuclear protein extracted from pCI-transfected cells. The involvement of NF-κB in the activation of the iNOS promoter mediated by HCV core protein was further demonstrated by cotransfecting HepG2 cells with p1iNOS-CAT and pHCV-Co plasmids and increasing amounts of the IκB expression vector pRSV-IκB. CAT analysis of transfected cells showed that CAT activity decreased as the amount of transfected pRSV-IκB plasmid increased, confirming that HCV core protein transactivation of the iNOS transcription is mediated by NF-κB.

The pathological consequence of the increase in NO generation in hepatocytes during the course of a chronic HCV infection remains largely unknown. However, as mentioned earlier, it has been shown that the level of nitrated proteins which are stable end products resulting from the reaction of NO with the ortho position of aromatic amino acids, are increased in the liver of patients with chronic HCV infection, and that the levels of these nitrated proteins are correlated with the severity of liver damage (García-Monzón et al., 2000). This observation together with our findings suggests that one of the mechanisms by which HCV causes liver damage may be the activation of iNOS transcription and NO generation by the HCV core protein. If this is true, therapies directed to inhibit selectively hepatic iNOS activity may be of benefit in the treatment of chronic HCV infection.

On the other hand, it has been reported that the HCV core protein induces immortalization of primary human hepatocytes (Ray et al., 2000) and promotes development of hepatocellular carcinoma in transgenic mice (Moriya et al., 1998). In this regard, it has been demonstrated that NO causes DNA damage and mutations in cells (Nguyen et al., 1992), inhibits the enzymes responsible for DNA repair (Laval and Wink, 1994, p. 38; Jaiswal et al., 2001) provokes conformational and functional changes in the tumor suppressor protein p53 (Camels et al., 1997) and inhibits apoptosis (Kim et al., 1997). Thus, it may be hypothesized that HCV core protein may contribute to the

hepatocarcinogenesis caused by HCV by inducing the generation of NO.

Finally, it has been shown that NO inhibits hepatitis B virus replication in transgenic mice in a non-cytolytic way (Guidotti et al., 2000). Whether NO is also able to diminish HCV replication, reducing the expression of viral proteins in the infected hepatocytes, thus allowing the virus to escape to the recognition of the host immune system, deserves future research.

In conclusion, in this report, we have demonstrated that the HCV core protein activates hepatic iNOS transcription through the activation of the NF-κB signaling pathway. The pathological consequences of this finding, as well as if selective inhibition of hepatic iNOS activity may be of benefit in the treatment of chronic HCV infection, should be studied in the future.

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