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Journal of Hepatology 45 (2006) 271–279
Journal of Hepatology
www.elsevier.com/locate/jhep

Oxidative stress inhibits IFN-α-induced antiviral gene expression by blocking the JAK–STAT pathway

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***Background/Aims*: Unresponsiveness to IFN-α is common in chronic hepatitis C. Since conditions associated with an increased oxidative stress (advanced age, steatosis, fibrosis, iron overload, and alcohol consumption) reduce the likelihood of response, we hypothesized that oxidative stress may affect the antiviral actions of IFN-α.**

***Methods*: We examined in a human hepatocellular carcinoma cell line (Huh-7) the effect of hydrogen peroxide (H_2O_2), as a generator of oxidative stress, on the IFN-α signaling pathway.**

***Results*: Pretreatment of Huh-7 cells with 0.5–1 mM H_2O_2 resulted in the suppression of the IFN-α-induced antiviral protein MxA and of IRF-9 mRNA expression. The reduced expression of these genes was associated to H_2O_2-mediated suppression of the IFN-α-induced assembly of signal transducer and activator of transcription (STAT) factors to specific promoter motifs on IFN-α-inducible genes. This was accomplished by preventing the IFN-α-induced tyrosine phosphorylation of STAT-1 and STAT-2 through the inactivation of the upstream receptor associated tyrosine kinases, JAK-1 and Tyk-2. The suppression was fast, occurring within 5 mins of pretreatment with H_2O_2, and did not require protein synthesis.**

***Conclusions*: In conclusion, oxidative stress impairs IFN-α signaling and might cause resistance to the antiviral action of IFN-α in chronically HCV infected patients with high level of oxidative stress in the liver.**

***Keywords*: Hydrogen peroxide; IFN-α; STAT; JAK-1; Tyk-2**

1. Introduction

Interferons (IFN)-α/β are crucial components of the early host response against virus infections. The biological activities of IFNs are mediated by a conserved signal transduction pathway [1]. Upon ligand binding to the IFN-α/β receptor, which lacks intrinsic kinase activity, the receptor-associated *Janus* family kinases, JAK-1 and Tyk-2, autophosphorylate on tyrosine residues and activate the cytoplasmic signal *transducers* and *activators* of transcription proteins, STATs. Phosphorylated STATs translocate to the nucleus to activate transcription of many target genes [1].

In recent years, a considerable body of evidences has accumulated demonstrating that the balance of the oxidative and reductive potentials within the cell (cellular

Received 22 December 2005; accepted 17 January 2006; available online 9 March 2006
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Abbreviations: AP-1, activating protein-1; DEM, diethyl maleate; EMSA, electrophoretic mobility shift assay; H_2O_2, hydrogen peroxide; HCV, hepatitis C virus; IFN, interferon; IFNAR1, α subunit of the IFN-α/β receptor; IRF-9, interferon regulatory factor-9; ISGF-3, IFN-stimulated gene factor-3; NF-kB, nuclear factor-kB; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription.
† Cinzia Fionda is recipient of a fellowship from the Italian Foundation for Cancer Research (FIRC).

0168-8278/$32.00
doi:10.1016/j.jhep.2006.01.037

redox state) can have profound consequences on signal transduction pathways, and the transduced signal can be sensitively modulated, or even abrogated, by prevalent cellular conditions [2–4].

The intracellular redox balance is tightly controlled in most cell types, but it can be altered by the production of reactive oxygen species (ROS) during pathologic conditions such as chronic inflammatory diseases [5].

IFNs are currently used as pivotal treatment in chronic hepatitis C. However, most patients are poorly responsive to IFNs [6,7]. Mechanisms underlying resistance to IFNs are not clear, and both viral and host factors have been proposed [6–11]. Clinical data indicate that patients with chronic hepatitis C who have conditions associated with elevated oxidative stress, such as advanced age [12], hepatic steatosis [13], liver fibrosis [14], iron overload [15] and alcohol consumption [16], have a reduced likelihood of response to IFN treatment.

Here, we tested the hypothesis that increased oxidative stress may be involved in an inhibitory pathway of the IFN-α activity.

The relationship between ROS and STATs was poorly explored [17,18] and to date, no study has investigated the effects of oxidants on STATs regulation in liver cells, as important potential targets for oxidants during chronic hepatitis. To understand whether IFN signalling may be influenced by oxidative stress, we investigated the effects of oxidants on IFN-α-induced activation of the JAK/STAT pathway in a human hepatocellular carcinoma cell line, and we demonstrated that oxidative stress inhibits IFN-α-induced STAT activation and antiviral protein expression.

2. Materials and methods

2.1. Cell lines, reagents, and treatments

Huh-7, human hepatoma cells were cultured in Dulbecco's modified Eagle's culture medium, supplemented with 10% fetal calf serum (Euroclone, UK), antibiotics, and glutamine (Euroclone, UK). All the experiments were performed with cells at low (20–30%) confluence. In all experiments, after pretreatment with various agents, culture medium was changed and stimulation with IFN-α was done in fresh culture medium. Recombinant human IFN-α (Intron A) was from Schering-Plough (Kenilworth, NJ). H_2O_2 and diethyl maleate were purchased from Sigma (St Louis, MO). Antibodies to phospho-Stat-1 (Tyr^{701}), phospho-JAK-1 ($Tyr^{1022/1023}$), phospho-Tyk-2 ($Tyr^{1054/1055}$), and JAK-1 were purchased from Cell Signaling (Beverly, MA). Antibody to phospho-Stat-2 (Tyr^{689}) was from Upstate Biotechnology (Lake Placid, NY). Antibodies to Stat-1, Stat-2, Tyk-2, IFNAR1, and phospho-IFNAR1 (Tyr^{466}) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to IFNAR1 (AA3 mAb) [23] was kindly provided by Dr Sandra Pellegrini (Institut Pasteur, Paris, France).

2.2. RNA isolation and RT-PCR

Total RNA was extracted from cells using Trizol® reagent (Life Technology, Grand Island, NY). One to two micrograms of total RNA were reverse-transcribed (Promega, Madison, WI), and aliquots were used in subsequent PCR reactions. Primer sets are as follows: MxA sense, 5′-GCTACACACCGTGACGGATATGG-3′ and MxA antisense, 5′-CGAGCTGGATTGGAAAGCCC-3′; 2′,5′-OAS sense, 5′-ATT GACAGTGCTGTTAACATCATC-3′ and 2′,5′-OAS antisense, 5′-AGATCAATGAGCCCTGCA TAAACC-3′; IRF-9 sense, 5′-CAAGCAGGACTTCCGGGAGG-3′; IRF-9 antisense, 5′-CTTCCTGTGGCTCAGGGCTG-3′; β-actin sense 5′-GTGGGGC GCCCCAGGCACCA-3′ and β-actin antisense, 5′-CTCCTTAATGT CACGCACGATTTC-3′. Semiquantitative PCR conditions were optimized to obtain reproducible and reliable amplification within the logarithmic phase of the reaction.

2.3. DNA transfection and luciferase assay

Huh-7 cells were transfected by electroporation using the pGAS-TA-Luc plasmid (Clontech Laboratories, Palo Alto, CA), containing the luciferase reporter gene under the control of two copies of a STAT-1 enhancer element, located upstream of a minimal TATA-box sequence of the Herpes Simplex Virus thymidine-kinase promoter. About 4×10^6 cells were transfected by electroporation, at 200 mV and 950 μF, with 15 μg of reporter plasmid. Cells were then split into six 60-mm cell culture dishes in complete culture medium. After 24 h, cells were treated with different stimuli and harvested for luciferase assay by four cycles of rapid freezing and thawing. Protein concentration was quantified by the BCA method (Pierce, Rockford, IL). Luciferase activity was read using the luciferase assay system (Promega, Madison, WI) following the manufacturer's instructions.

2.4. Whole cell extract

After treatments, cells were resuspended in lysis buffer (30 mM Tris pH 8, 150 mM NaCl, 5 mM EDTA, 1% Triton, 10% glycerol) containing 20 mM β-glycerophosphate, 1 μM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 0.5 mM dithiotreithol, 1 μg/mL leupeptin, and 1 μg/mL aprotinin. Lysates were pre-cleared by centrifugation and protein concentration was determined as described above.

2.5. Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described in [19], using whole cell extracts (10 μg). Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). The double-strand probes were end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. The following double-strand oligomers were used as specific labeled probes or cold competitors: the Stat binding site, SIE-m67 (the high affinity mutated serum-induced element) 5′-GTCGACATTTCCCGTAAATCGTC GA-3′, and OCT (human histone H2b, −60 to −35 bp), 5′-agCT CTTCACCTTATTTGCATAAGCGAT-3′.

2.6. Western blot analysis

Forty to fifty micrograms of whole-cell extracts were run on 12% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NJ) and blocked in 3% milk in TBST. Immunoreactive bands were visualized on the nitrocellulose membranes, using horseradish-peroxidase-coupled goat anti-rabbit or anti-mouse immunoglobulins and the ECL detection system (Amersham, Arlington Heights, IL), following the manufacturer's instructions.

2.7. Flow cytometry

Surface IFNAR1 levels were monitored by incubating cells with 10 μg/mL of AA3 mAb [20], followed by incubation with 10 μg/mL of biotinilated anti-mouse IgG Abs and with streptavidin–phycoerythrin. Cells were analyzed with a FACScan flow cytometer (Becton

Dickinson). Assessment of cell viability and apoptosis was performed by propidium iodide/annexin V staining (Bender MedSystems, Vienna, Austria, Europe).

3. Results

3.1. H_2O_2 inhibits IFN-α-stimulated gene expression and transactivation

To determine the effect of oxidative stress on IFN-α-stimulated gene expression, we examined the activity of H_2O_2 on IFN-α-induced gene expression in Huh-7 human hepatoma cells. The antiviral gene MxA and IRF-9 (p48, part of the IFN-stimulated gene factor-3 transcriptional complex) were here used as indicators of IFN-α-induced gene expression. RNA was prepared from Huh-7 cells and the induction of IFN-α-stimulated genes was analyzed by RT-PCR. Treatment of Huh-7 cells with IFN-α caused an increase of MxA and IRF-9 mRNA (Fig. 1A), but pretreatment with H_2O_2 (0.5–1 mM) reduced or abrogated IFN-α-induced expression of these genes (Fig. 1A). In all experiments, expression levels of β-actin were not affected, indicating that the effect of H_2O_2 on IFN-α-induced genes was not caused by a nonspecific toxicity. H_2O_2 alone did not affect the expression of MxA and IRF-9 (data not shown).

The IFN-α-induced gene expression is under the control of STATs, which once activated, translocate to the nucleus and activate transcription [1]. To assess whether STAT activation could be involved in the inhibitory activity of H_2O_2 on IFN-α-induced genes, we tested the activity of a luciferase reporter vector driven by STAT elements, in transient transfection experiments in Huh-7 cells. The IFN-responsive reporter yielded a mean of about fourfold induction of luciferase activity upon IFN-α treatment (Fig. 1B), while no induction was observed with H_2O_2 alone (data not shown). Pretreatment with H_2O_2 reduced the induction up to less then twofold, consistent with the involvement of the STAT pathway in the inhibitory activity of H_2O_2 (Fig. 1B).

3.2. Oxidative stress inhibits IFN-α-induced DNA-binding of STAT-1

To examine the mechanism(s) by which H_2O_2 inhibits IFN-α-induced transcriptional activity, the effect of H_2O_2 on IFN-α signaling pathway was investigated. EMSA were used to determine whether the H_2O_2 inhibition of IFN-α-stimulated transcriptional activity was due to an inability to form ISGF3 or STAT-1 homodimers. In EMSA experiments utilizing a ^{32}P-labeled ISRE probe, we could not detect ISGF3 complexes in

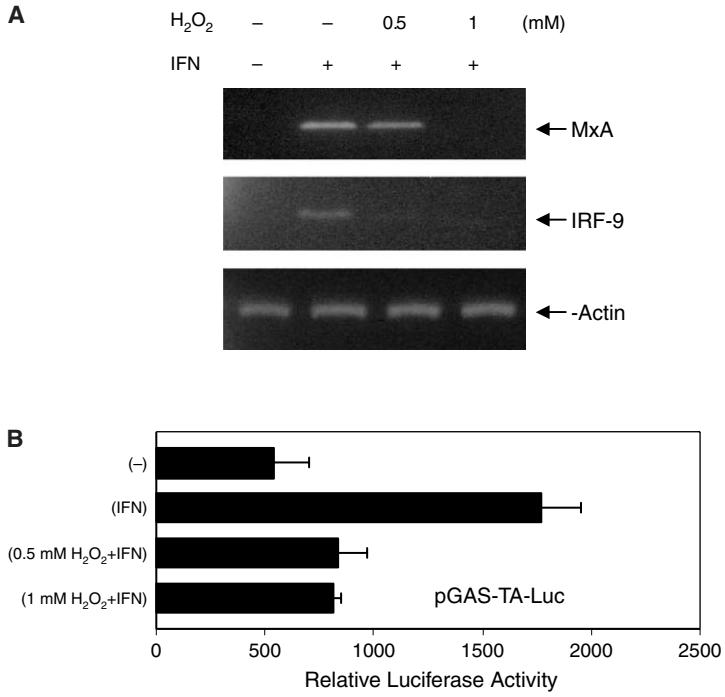

Fig. 1. H_2O_2 inhibits IFN-α-induced gene expression. (A) Huh-7 cells were treated with 0.5 and 1 mM H_2O_2 for 30 min, followed after wash with fresh culture medium, by stimulation with IFN-α (200 U/mL) for 2 h. Total RNA was prepared and subjected to RT-PCR, using primers for MxA, IRF-9 and β-actin. One experiment representative of three is shown. (B) Huh-7 cells were transfected with pGAS-TA-Luc reporter construct. Cells were pre-treated for 30 min with 0.5 and 1 mM H_2O_2 followed by treatment with IFN-α (200 U/mL) for 30 min in fresh culture medium. Culture medium was then replaced and cells were collected after 6 h to prepare cell extracts for luciferase activity. Relative luciferase activities are from an average of three experiments. All transfections were performed in duplicate for each experiment. Error bars represent standard deviations.

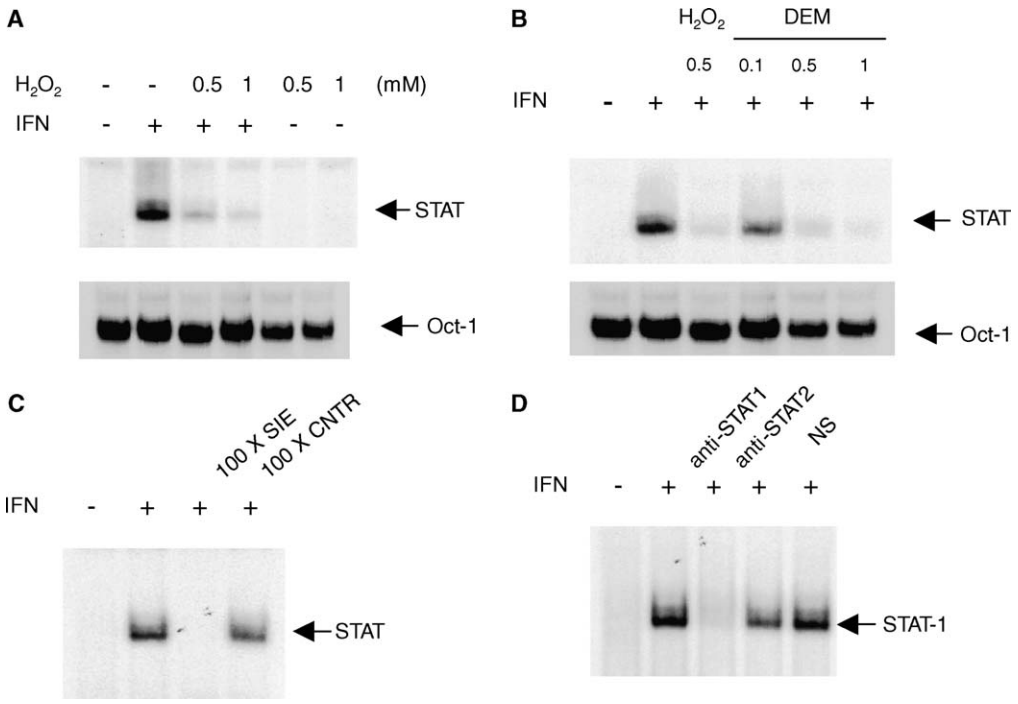

Fig. 2. H_2O_2 inhibits IFN-α-induced STAT-1 homodimer formation. (A) Huh-7 cells were pre-treated with 0.5 and 1 mM H_2O_2 for 30 min, followed after wash with fresh culture medium, by a 30-min stimulation with IFN-α (200 U/mL) in normal culture medium. Cell extracts were then prepared for EMSA. One experiment representative of three is shown. (B) DEM inhibits IFN-α-stimulated STAT activation in the absence of H_2O_2. Huh-7 were pre-treated with different doses of DEM for 1 h, and then, after wash with fresh culture medium, treated with IFN-α (200 U/mL) for 30 min. One experiment representative of three is shown. (C) Competition experiments with 100-fold molar excess of unlabeled m67 oligonucleotides (SIE) and a non-specific control oligonucleotides (OCT) confirmed the specificity of the complex. (D) Supershift analysis with antibodies to STAT-1 verified that the probe binds STAT-1 homodimers.

Huh-7 treated with IFN-α (data not shown). However, in this cell line, the basal level of p48 (IRF-9) is limiting (Fig. 1A), thus hampering the early assembly of the ISGF3 complex. Therefore, the ability of IFN-α to stimulate the formation of STAT dimers was tested by EMSA experiments utilizing the m67 (high-affinity mutated serum-induced element) as probe. The m67 element binds to IFN-α-activated STAT (Fig. 2A). Thirty minutes pretreatment of Huh-7 cells with H_2O_2 inhibited IFN-α-induced STAT binding in a concentration-dependent manner, whereas H_2O_2 itself had no effects on STAT binding (Fig. 2A). H_2O_2 inhibition of IFN-α activity was evident at 500 μM, while a complete inhibition of STAT binding occurred at 1 mM (Fig. 2A).

To evaluate whether the inhibitory activity of H_2O_2 was IFN-α-specific or whether this effect was evident for other cytokines activating the JAK/STAT transduction pathway, we assessed the ability of H_2O_2 to inhibit IFN-γ-induced STAT activation. A 5 min pretreatment of HUH-7 cells with with 1 mM H_2O_2 resulted in an inhibition of STAT activation assessed by EMSA (data not shown), demonstrating that in this cell line the prevalent effect of H_2O_2 is the inhibition of the JAK/STAT pathway.

Pretreatment of cells with the glutathione chelating agent diethyl-maleate (DEM) produced the same inhibitory effect of H_2O_2 on IFN-α-induced STAT activation (Fig. 2B), suggesting that the inhibition of the JAK–STAT pathway is the result of a general increase of the oxidative stress in the cells. As a control for equal proteins loading, the same amount of proteins was run in the presence of an Octamer factor(s)-specific probe (Fig. 2A and B).

Cold competition experiments confirmed the specificity of the complex (Fig. 2C). Supershift analysis with antibodies to Stat-1 showed that the m67 probe binds STAT-1 homodimers (Fig. 2D).

To determine the rate of onset of the inhibitory effect of H_2O_2, cells were exposed to 1 mM H_2O_2 for various times before IFN-α stimulation. As shown in Fig. 3A, almost complete inhibition of STAT-1 activation was evident after a 5 min pre-incubation of cells with H_2O_2.

We then investigated whether this effect was reversible. Huh-7 cells were treated with H_2O_2 for 5 min after which H_2O_2 was removed and cells were left in normal growth conditions and stimulated with IFN-α after 24 h. As shown in Fig. 3B, the inhibitory effect of H_2O_2 significantly subsided (lanes 7 and 8), although the IFN-α-induced STAT-1 binding remained lower than control cells (lane 2), suggesting that the IFN-α signaling is not permanently compromised, and that cells can recover this function if oxidative stress is removed.

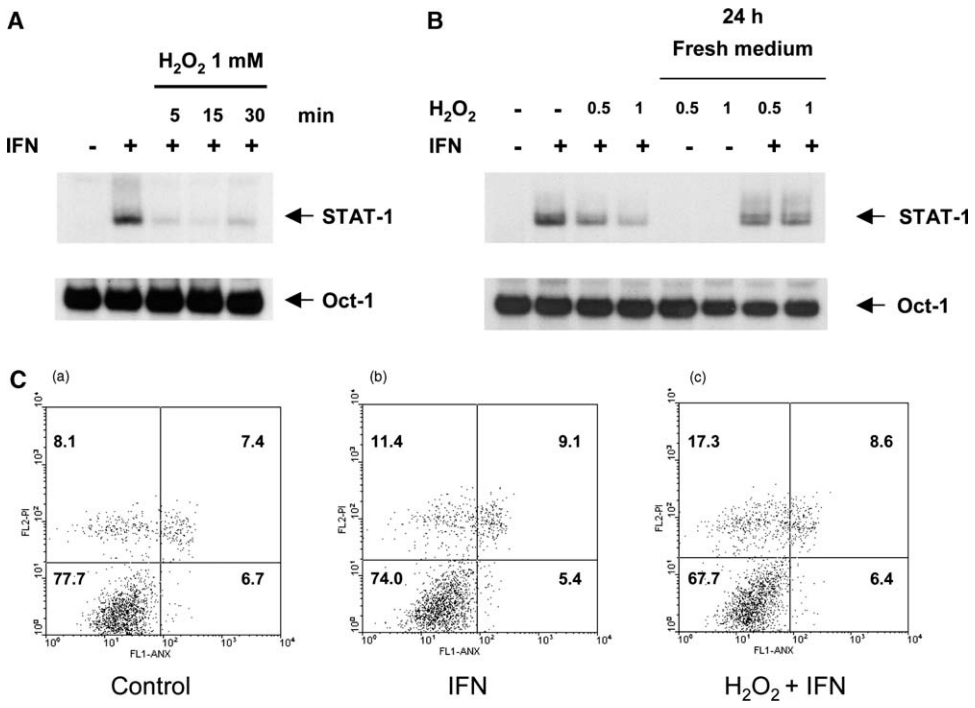

Fig. 3. Inhibition of IFN-α-induced STAT-1 by H_2O_2 is rapid and reversible. (A) Huh-7 were treated with H_2O_2 for 5, 15, and 30 min and then, after wash with fresh culture medium, incubated with IFN-α (200 U/mL) for 30 min in normal culture medium. One experiment representative of three is shown. (B) Huh-7 cells were treated with H_2O_2 for 5 min and then, after wash with fresh culture medium, incubated with IFN-α (200 U/mL) for 30 min in normal culture medium (lanes 2–4), or, after removal of H_2O_2, cultured in normal culture medium for additional 24 h (lanes 5–8), and then stimulated with IFN-α for 30 min (lanes 7 and 8). One experiment representative of three is shown. (C) HUH-7 cells were pre-treated with 1 mM H_2O_2 for 5 min and then, after wash with fresh culture medium, incubated with 200 U/mL IFN-α for 30 min. The cells were stained with Annexin V-FITC and PI. Percent of single- and double-positive cells is displayed in each panel. The experiment is representative of three independent experiments all displaying similar results.

The inhibition of IFN-α-induced STAT-1 activation was not due to a significant decrease in cell viability. Quantitative discrimination between the viable, early apoptotic, and necrotic subpopulations of H_2O_2 pre-treated cells was obtained by fluorescence-activated cell sorting analysis done in parallel with EMSA experiments (Fig. 3C). Although the ability of the cells to activate STATs in response to IFN-α was significantly impaired, cells pre-treated with 1 mM H_2O_2 retained approximately 87% of their original viability as judged by propidium iodide exclusion and a lack of surface-accessible annexin V.

3.3. H_2O_2 inhibits IFN-α-stimulated tyrosine phosphorylation of Stat-1 and Stat-2

As outlined above, STAT proteins are activated by phosphorylation of a single tyrosine residue immediately

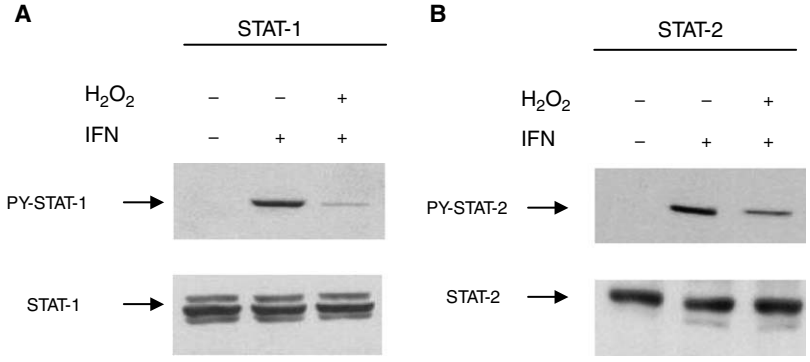

Fig. 4. H_2O_2 inhibits IFN-α-stimulated tyrosine phosphorylation of STAT-1 and STAT-2. Huh-7 were pre-incubated with or without 1 mM H_2O_2 for 5 min and then, after wash with fresh culture medium, incubated with 200 U/mL IFN-α for 30 min. Whole cell extracts were subjected to Western blotting using antibodies specific to phospho-tyrosine (PY)-STAT-1 and STAT-1 (A) or to PY-STAT-2 and STAT-2 (B). One experiment representative of three is shown.

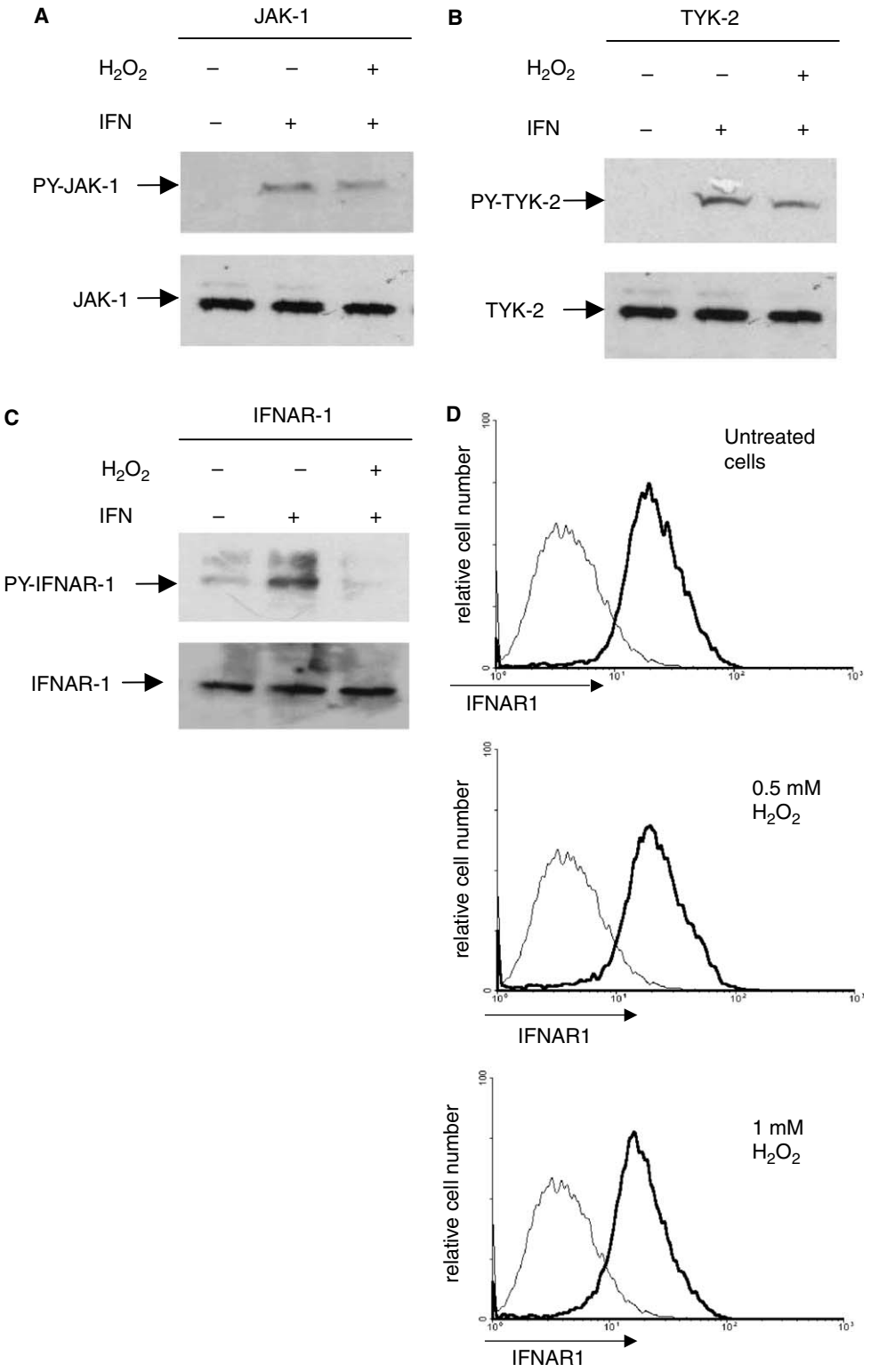

Fig. 5. H_2O_2 inhibits IFN-α-stimulated tyrosine phosphorylation of JAK-1, Tyk-2, and the α-subunit of the IFN-α/β receptor (IFNAR1), without lowering cell surface levels of IFNAR1. Huh-7 were incubated with or without 1 mM H_2O_2 for 5 min and then, after wash with fresh culture medium, incubated with 200 U/mL IFN-α for 15 min. Whole cell extracts were subjected to Western blotting using antibodies specific to phosphorylated JAK-1 (PY-JAK-1) and JAK-1 (A), to phosphorylated Tyk-2 (PY-Tyk-2) and Tyk-2 (B), and to phosphorylated IFNAR1 (PY-IFNAR1) and IFNAR1 (C). One experiment representative of three is shown. (D) The level of cell surface IFNAR1 was determined by flow cytometry using the AA3 mAb. Thin lines represent staining with the secondary biotinilated anti-mouse IgG antibody. IFNAR1 surface levels of cells left untreated (top panel; bold line, mean 24.85), or treated with 0.5 (middle panel; bold line, mean 24.10) or 1 mM H_2O_2 for 30 min (bottom panel; bold line, mean 21.03) are shown. One experiment representative of three is shown.

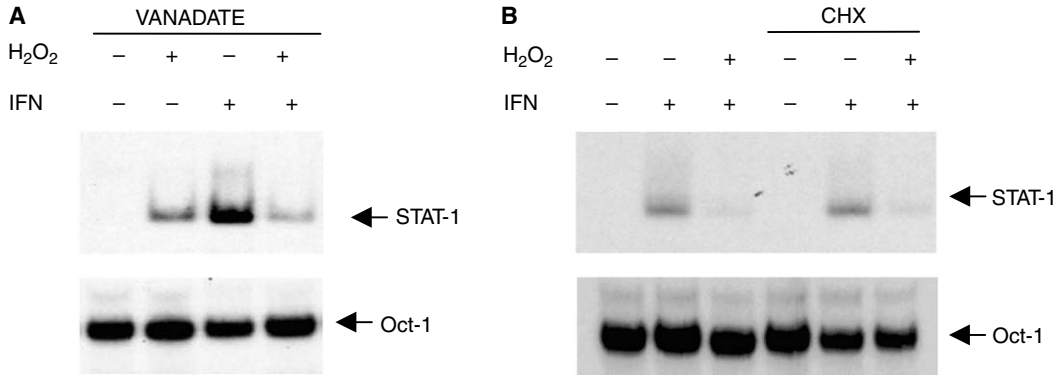

Fig. 6. H_2O_2 inhibits IFN-α-stimulated STAT activation is not dependent on tyrosine phosphatase activity and new protein synthesis. (A) Huh-7 cells were incubated with vanadate 100 μM for 30 min. H_2O_2 was then added in new fresh medium to some samples for 5 min, prior to the incubation with IFN-α for 30 min in new culture medium. Cell extracts were then subjected to EMSA. One experiment representative of three is shown. (B) Huh-7 cells were incubated with cycloheximide (CHX, 20 μg/mL) for 30 min, then treated with H_2O_2 for 5 min followed by a 30-min stimulation with IFN-α (200 U/mL) in new culture medium. Cell extract were then subjected to EMSA. One experiment representative of three is shown.

downstream of the SH2 domain. To test whether the H_2O_2-mediated inhibition of STAT-DNA binding was caused by impaired STAT activation, IFN-α-induced tyrosine phosphorylation of STAT-1 and STAT-2 was examined. Pretreatment of Huh-7 cells with H_2O_2 inhibited IFN-α-stimulated tyrosine phosphorylation of both STAT-1 (Fig. 4A) and STAT-2 (Fig. 4B). The blots were reprobed with their respective STAT-specific antisera to ensure that equal amounts of proteins were present in each sample (Fig. 4A and B).

3.4. H_2O_2 inhibits IFN-α-stimulated tyrosine phosphorylation of JAK-1, Tyk-2, and the α subunit of the IFN-α/β receptor (IFNAR1), without lowering the cell surface expression of IFNAR1

To define whether H_2O_2 suppression of IFN-α-induced tyrosine phosphorylation of STAT-1 and STAT-2 could be due to inactivation of upstream kinases, the effect of H_2O_2 on JAK-1 and Tyk-2 was examined. Pretreatment of Huh-7 with H_2O_2 significantly inhibited IFN-α-induced tyrosine phosphorylation of both JAK-1 (Fig. 5A) and Tyk-2 (Fig. 5B), demonstrating that the block of IFN-α signaling is present at the level of receptor-associated tyrosine kinases. Equal amounts of both JAK-1 and Tyk-2 were present in all samples, ruling out the possibility that incubation of Huh-7 with H_2O_2 induced a selective degradation of these proteins (Fig. 5A and B).

Since IFNAR1 has been shown to be rapidly tyrosine phosphorylated as result of exposure of cells to IFN-α [1], we examined the effect of H_2O_2 on the receptor phosphorylation, and we found that H_2O_2 had the same inhibitory effect on this outcome (Fig. 5C). Western blot analysis revealed no change in the relative level of expression of IFNAR1 after H_2O_2 treatment, and cytofluorimetric analysis using an antibody specific to IFNAR1, showed no H_2O_2-mediated reduction of IFNAR1 cell surface expression (Fig. 5D), ruling out the possibility that decreased IFNAR1 cell surface expression could account for the inhibitory action of H_2O_2 on IFN signaling.

3.5. Activation of protein tyrosine phosphatases (PTPs) and protein synthesis are not involved in H_2O_2-mediated inhibition of IFN-α-activated signal transduction

From the results presented above, it appeared that H_2O_2 treatment inhibited tyrosine phosphorylation of the IFN-α receptor associated tyrosine kinases. A possible mechanism to explain this effect could be that H_2O_2 activates tyrosine phosphatases, which in turn dephosphorylate activated JAK-1 and Tyk-2. In this case, the incubation of cells with the tyrosine phosphatase inhibitor vanadate should reverse the inhibitory effect of H_2O_2. Huh-7 cells were incubated for 30 min with vanadate, prior to the addition of H_2O_2 for 5 min, and IFN-α for further 30 min. While no activation of STAT complexes was visualized after incubation with H_2O_2 (data not shown) or vanadate alone, the simultaneous exposure to both compounds resulted in activation of STAT-1 (Fig. 6A, lane 2), due to the enhanced inhibitory effect of vanadate on basal active PTPs in the presence of peroxides [21]. However, pre-incubation of Huh-7 with vanadate did not prevent the inhibition by H_2O_2 of IFN-α-induced formation of STAT complexes (Fig. 6A, lane 4), ruling out the possibility that the inhibitory action of H_2O_2 could be related to activation of PTPs.

An important mechanism responsible for down-regulation of the JAK–STAT pathway is the induction of newly synthesized inhibitory proteins [22]. Therefore, we investigated whether the H_2O_2-mediated inhibition of STAT activation was affected by blocking new

protein synthesis. As shown in Fig. 6B, blocking new protein synthesis with cycloheximide did not abolish H_2O_2-mediated suppression of IFN-α-activated STAT, suggesting that rapid inhibition of IFN-α signaling is not due to de novo synthesis of inhibitory proteins.

4. Discussion

We demonstrate that, in a human hepatocellular carcinoma cell line, oxidative stress elicited by H_2O_2 or DEM, inhibits IFN-α-induced activation of STAT-1 and STAT-2 that results in the reduction of STAT-dependent gene transcription. The effect of the oxidative stress on STATs is accomplished by inhibition of IFN-α-induced activation of JAK-1 and Tyk-2. The inhibitory effect of oxidative stress on the JAK/STAT pathway is rapid, and is not dependent on new protein synthesis. The inhibition of IFN-α signalling by H_2O_2 is reversible upon removal of H_2O_2, suggesting that this inhibition was not due to irreversible cell damage.

Different transcription factors are modulated by oxidative stress, such as NF-κB or AP-1 [3,4]. To date, the only published studies on the relationship between STAT factors and oxidative stress have reported that H_2O_2 is able to activate STAT-3 and slightly STAT-1, in rat fibroblasts and in a human epidermoid carcinoma cell line [17], and STAT-3 in human lymphocytes pretreated with aminotriazole, a catalase inhibitor [18]. These studies have not considered the effect of the cytokines after exposure of the cells to oxidants. Our data demonstrate that in a human hepatocellular carcinoma cell line, endogenously produced or added H_2O_2 does not activate any STAT factors, and inhibits IFN-α-induced STAT-1 and STAT-2 activation. These findings are similar to those reported in some studies on other transcription factors, such as NF-κB [3,23]. In this regard, it was demonstrated that NF-κB could be directly activated by oxidative stress; however, this activation is not observed in all cell lines, but is strictly cell type dependent [3]. Moreover, the cytokine-induced activation of NF-κB can be also inhibited in the presence of H_2O_2 in several cell lines [23]. Our data suggest that, similarly to what reported for NF-κB, also the regulation of the JAK/STAT by oxidants is cell type dependent, and that the cytokine-induced activation of this pathway could be widely modulated by the oxidative microenvironment.

Our data provide evidence that the inhibition of STAT signaling is accompanied by the inhibition of the receptor-associated kinases JAK-1 and Tyk-2, suggesting that these two kinases could be a possible target of oxidative stress-mediated inhibition. We found a normal expression level of IFN-α receptor on cell surface in cells pre-treated with H_2O_2, indicating that oxidative stress does not prevent activation of the JAK-1 and Tyk-2 by decreasing the expression of IFN-α receptor on cell surface.

Tyrosine phosphorylation of proteins is dependent on the balance between kinases and PTPs within the cell. We tested the hypothesis of an involvement of PTPs in the inhibitory effect of oxidative stress on STAT activation, through a dephosphorylation of the activated proteins of the JAK/STAT pathway. We observed that the inhibitor of PTPs, vanadate, was not able to revert the inhibitory effect of H_2O_2 on IFN-α-induced STAT activation, therefore, the inhibitory effect of oxidative stress on JAK/STAT activation can not be due to increased activity of PTPs induced by oxidative stress.

Duhè et al. reported that JAKs are redox-sensitive enzymes [24]. The autokinase activity of JAKs can be directly and reversibly inhibited by thiol oxidants, such as nitric oxide [24]. Accordingly, a possible explanation for the observed inhibitory effect of oxidative stress on IFN-α signaling in Huh-7 cells, could be that oxidative stress, caused by H_2O_2 or DEM, directly inactivates the receptor associated kinases Jak-1 and Tyk-2, by oxidizing their intramolecular thiol groups.

The differences between our results and those reported from other groups [17,18] on the redox regulation of STAT components can be reconciled by the consideration that both protein tyrosine kinases and protein tyrosin phosphatases, which together influence the activity of the JAK/STAT pathway, are redox sensitive enzymes. Similarly to the IFNAR1-associated protein kinases, the PTPs are inactive when the thiol group in the catalytic site is oxidized to a disulfide. Therefore, the net outcome of redox perturbation of IFN-α signal transduction will depend on the net balance of active and inactive forms of kinases and the counteracting phosphatases. An essential determinant of this balance could be the chemical nature and the abundance of the intracellular redox modifiers, which widely vary among the different cell types, thus explaining the cell specificity activatory or inhibitory effect on the JAK/STAT pathway [25].

Almost all the cytokines, released during inflammatory processes by the cells of immune system, activate STAT factors. Interestingly, during the inflammatory response, high levels of ROS are generated [5]. Considering the role of oxidative/reductive events within the cells in conditioning signalling, it is important to understand the function of the JAK/STAT pathway under conditions similar to those occurring during pathological states. In this work, we provide evidences that oxidative stress suppresses IFN-α-induced STAT activation in human hepatocellular carcinoma cells. The results suggest that under inflammatory conditions or other disease states, where H_2O_2 or other oxidants are formed, the activation of the JAK/STAT pathway could become depressed in certain cell types. It has been reported that reactive oxygen species (ROS) are elevated in the liver

during chronic hepatitis C, due both to the direct contribution of the virus and to the inflammatory process [5,26–29]. Therefore, the oxidative inhibition of the JAK/STAT pathway could be one of the mechanisms to explain the resistance to the antiviral action of IFN-α in HCV chronically infected patients with conditions associated with high level of oxidative stress in the liver such as advanced age, steatosis, fibrosis or iron overload. Our findings might support the concept that the antioxidants, which are able to counteract ROS, could be investigated as potential adjunct therapeutic agents for chronic hepatitis C. Clearly, further studies are warranted to confirm this hypothesis, and to elucidate the exact mechanism of inhibition of the JAK/STAT signaling by oxidants in hepatic cells.

Acknowledgements

The authors thank Sandra Pellegrini for the kind gift of AA3 monoclonal antibody, Dr Mario Melis for his assistance during flow cytometric assays, Professor Massimo Levrero, MD, Dr Elisabetta Pace and Dr Mark Gjomarkaj for their helpful revision of the manuscript.

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