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Up-regulation of the interferon-inducible IFI16 gene by oxidative stress triggers p53 transcriptional activity in endothelial cells

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Abstract: Reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), induces injury of endothelium in a variety of pathophysiological conditions, such as inflammation, aging, and cancer. In our study, we characterized the signaling pathway linking oxidative stress induced by sublethal concentrations of H₂O₂ to p53 in primary human endothelial cells through the interferon (IFN)-inducible gene IFI16. Induction of IFI16 by H₂O₂ was concentration- and time-dependent (maximum at 50 μM, 6 h after treatment) and down-regulated by pretreatment with N-acetyl-L-cysteine, which acts as an antioxidant. This pathway is a general response to ROS and not specific to H₂O₂ treatment, as two other ROS-generating compounds, i.e., S-nitroso-N-acetylpenicillamine and tert-butyl hydroperoxide, were equally capable to induce IFI16. Moreover, IFI16 up-regulation is a result of protein accumulation, as expression of corresponding mRNA, assessed by real-time polymerase chain reaction, was not affected. To investigate the mechanism of IFI16 accumulation, cells were incubated for 6 h in the presence of H₂O₂ or IFN-β, and then cycloheximide was added to inhibit further protein synthesis. The half-life of IFI16 protein was found to be significantly increased in H₂O₂-treated cells compared with IFN-β-treated cells (t_{1/2}=120 min vs. >30 min in H₂O₂- vs. IFN-β-treated cells, respectively). An increase of IFI16 was accompanied by interaction with p53 phosphorylated at its N terminus, as shown by immunoprecipitation experiments. Moreover, binding to IFI16 resulted in its transcriptional activation as shown by an increase in the activity of a reporter gene driven by p53-responsive sequences derived from the p21^{WAF1} promoter, along with an increase in the p21 mRNA and protein levels. Altogether, these results demonstrate a novel role of IFI16 in the signal transduction pathway that leads to p53 activation by oxidative stress in endothelial cells. *J. Leukoc. Biol.* 77: 820–829; 2005.

Key Words: ROS · HUVEC · inflammation

INTRODUCTION

The human IFI16 gene, originally identified as a target of interferons (IFN)-α/β and γ, is a member of the HIN200 family, which includes myeloid nuclear differentiation antigen, absent in melanoma 2, IFIX in humans, and Ifi202a, Ifi202b, Ifi203, Ifi204, D3 genes in mice [1–4]. The findings that IFI16 protein and its mouse homologues p202 and p204 interact with p53, Rb, E2F, activated protein 1, and nuclear factor-κB proteins lead to the hypothesis that it may regulate the function of different transcription factors [2, 3, 5–11]. More recently, a role of IFI16 in the apoptosis pathway has been postulated by the findings that the PYRIN domain, which is commonly found among cell death-associated proteins, has been located in its N-terminal [12].

Since its original cloning, IFI16 has been considered specific for hematopoietic cells [13]. However, further investigation demonstrated that in normal adult human tissues, it is expressed in a highly restricted pattern in selected cells within certain organs [14, 15]. Prominent IFI16 expression is seen in vascular endothelial cells from blood and lymph vessels and in stratified squamous epithelia, suggesting its possible link to inflammation in addition to hematopoiesis. As IFI16 modulates transcriptional activation by p53, it may regulate endothelial and epithelial cell physiology through this molecular pathway. Consistent with this hypothesis, transduction of IFI16 into human umbilical vein endothelial cells (HUVEC) with a herpes simplex virus (HSV)-derived replication-defective vector efficiently suppressed endothelial migration, invasion, and formation of capillary-like structures in vitro [16]. In parallel, sustained IFI16 expression inhibited HUVEC cell-cycle progression, accompanied by significant induction of p53, p21, and hypophosphorylated pRb. These findings, with the observation that HUVEC, immortalized by human papillomavirus type 16 (HPV16) E6/E7 oncogenes, became resistant to antiangiogenic and antiproliferative activity caused by IFI16 overexpression, suggest a relevant role of IFI16 in the p53 transduction pathway.

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Reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide (H₂O₂), and hydroxyl radical, are generated in an enormous variety of normal metabolic processes, as they are released from several cell types [17–19]. Accumulation of excess ROS, which is toxic to cells, can cause oxidative stress, leading to damage to proteins, nucleic acid, and cell membranes. DNA damage triggers a variety of signaling pathways that lead to apoptosis or to DNA damage repair that is coupled with cell-cycle regulation [20–23]. The endothelium is highly sensitive to injury caused by ROS, including H₂O₂. ROS may cause endothelial activation, dysfunction, or death, depending on dose and exposure time. Low concentrations of ROS may activate intracellular signaling systems, which protect endothelial cells against inflammatory response. One junction of such pathways is controlled by p53, which functions as a potent transcription factor [20–23]. Normally, p53 is present at low levels in the cytoplasm as a result of its association with the murine double minute (MDM2) protein, a ubiquitin ligase that modifies p53 in its C-terminal domain [24, 25]. Cellular stress induces phosphorylation of its N-terminal domain and achieves p53 activation by inhibiting MDM2 binding and the nuclear export of p53 [24–28]. This phosphorylation has been correlated with enhanced induction and functional activation of p53 manifest as transcription of its target p21^{WAF1}.

Understanding the cellular responses to oxidative stress will provide useful insights into the mechanisms of many pathophysiological states, such as inflammation, ischemia-reperfusion, diabetes, hypertension, atherosclerosis, and sepsis [29]. In the present study, the regulation and functional role of IFI16 in response to oxidative stress was investigated. The results show that upon oxidative stress, IFI16 is up-regulated, and its interaction with p53 leads to transcriptional activation of p53-dependent genes, such as p21^{WAF1}.

MATERIALS AND METHODS

Cell cultures and reagents

HUVEC were isolated from cannulated human umbilical veins by treatment with collagenase and maintained in complete endothelial growth medium 2 (EGM-2; Clonetics, San Diego, CA), containing 2% fetal bovine serum, human recombinant vascular endothelial growth factor, basic fibroblast growth factor, human epithelial growth factor, insulin growth factor, hydrocortisone, ascorbic acid, heparin, and GA-1000 (gentamycin and amphotericin B, 1 µg/ml), according to the recommendations of the supplier. Each culture was used up to five population doublings. The cells were seeded into 100 mm culture dishes coated with 0.2% gelatin and grown under 5% CO₂ at 37°C in medium, renewed every 3–4 days. S-nitroso-N-acetylpenicillamine (SNAP) and tert-butyl hydroperoxide (tBHP) were purchased from Sigma-Aldrich (St. Louis, MO) and used as indicated at a concentration of 50 µM and 0.2 mM, respectively. Monoclonal antibodies (mAb) against p53 and p21^{WAF1} (p21) were purchased from Santa Cruz Biotechnology (CA), mAb against p53 Ser15, from Cell Signaling Technology (Beverly, MA), and mAb against human actin, from Chemicon International (Temecula, CA). Rabbit polyclonal monospecific anti-IFI16 antibodies were produced and purified as described previously [14]. For detection of intracellular reactive ROS level, 1 × 10³ HUVEC were seeded in four-well chamber slides and grown for 24 h in their optimal medium (as described above). For N-acetyl-L-cysteine (NAC) treatment, the pH of a 0.4 mol/L aqueous stock solution of NAC (Sigma-Aldrich) was adjusted to 7.4 with NaOH, sterilized by filtration, and diluted in EGM-2 medium to give a final concentration of 30 µmol/L. HUVEC were treated with 30 µmol/L NAC for 60 min before treatment with H₂O₂, as indicated in Results. IFI16 protein half-life

studies were performed by treating cells with H₂O₂ (50 µM) or IFN-β (1000 IU/ml) for 6 h. Cycloheximide (CHX; 120 µg/ml) was then added to inhibit further protein synthesis. Cells were harvested at 30 min and 1, 2, and 4 h after CHX addition. IFI16 protein expression levels were then analyzed by Western blot.

Retroviral expression vectors

The retroviral vector pBabe-puro, containing the HPV16 E6/E7 open-reading frame, was kindly provided by Massimo Tommasino (International Agency for Research on Cancer, Lyon, France). High-titer retrovirus containing supernatants (>5 × 10⁶ IU/ml) was generated by transient transfection of second-generation retrovirus producer Phoenix cells and used to infect the cells as described previously by Pear et al. [30]. After infection, HUVEC were selected in 0.2 µM/ml puromycin for 10 days and designated E6/E7 HUVEC.

Recombinant adenovirus preparation and HUVEC infection

The pAC-CMV IFI16, containing the human IFI16 cDNA linked to a FLAG-tag at the NH₂-terminal, was cotransfected together with pJM17 into 293 cells as described in the instruction manual (Microbix Biosystems Inc., Toronto, Ontario, Canada). Recombinant viruses from individual plaques, purified to homogeneity by three rounds of limiting dilution, were assessed for virus titer by plaque formation on the 293 complementing line with agar overlay as suggested by the manual. Recombinant AdvIF16 was tested for IFI16 expression by Western blotting, using an anti-FLAG antibody (Sigma-Aldrich). For cell transduction, postconfluent HUVEC were washed once with phosphate-buffered saline (PBS) and incubated with AdvIF16 or AdvLacZ at a multiplicity of infection (moi) of 50 in EGM. After 60 min at 37°C, the virus was washed off, and fresh, low-serum medium was added. Cells were cultured for 36 h before use in the experiments.

Plasmids and luciferase assay

p53RELuc and mutated p53 (mutp53)RELuc plasmids contain two copies of a wild-type p53 (wtp53) binding sequence (5'-CGAATTCGAACATGTCCGAACATGTTGAGATCTGCC-3') or two copies of a mutp53 binding sequence (5'-CCGAATTCGAAATTTCCGAATCCTTTGAGATCTGCC-3'), respectively, subcloned in the pGL3-promoter plasmid, harboring simian virus 40 (SV40) minimal promoter (Promega, UK).

Equal amounts (4 µg) of each reporter plasmid (harboring firefly Luc cDNA) along with 0.15 µg pRLSV40 plasmid (harboring Renilla Luc cDNA) included as a control for transfection efficiency were introduced into HUVEC cells by Lipofectamine Plus (Gibco, Grand Island, NY). Twenty-four hours later, cells were infected with recombinant AdvIF16 or AdvLacZ viruses and then treated or untreated with H₂O₂. After 24 h incubation, cells were assayed for chemiluminescence using the dual luciferase reporter system as suggested by the manufacturer's instructions (Promega). Chemiluminescence was measured using the Lumino luminometer (Strattec Biomedical Systems, Birkenfeld, Germany). For all cotransfection treatment studies, values were normalized by dividing values for firefly Luc expressed as relative light units by values obtained from *Renilla* Luc. The resulting luciferase activity is expressed as fold induction relative to basal levels measured in cells transfected with p53RELuc and infected with AdvLacZ, which was set at 1.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted with GenElute mammalian total RNA kit (Sigma-Aldrich). Total RNA (1 µg) was reverse-transcribed, after 10 min of denaturation at 70°C, in 20 µl reaction mixture containing 500 µM of each deoxy-unspecified nucleoside 5'-triphosphates, 2.5 µM random nonamers, 20 U RNase inhibitor, and 20 U enhanced avian RT (all from Sigma-Aldrich). The reaction mixture was incubated at 25°C for 15 min and 45°C for 50 min. Real-time RT-PCR was performed on a GeneAmp 7000 sequence detection system (Applied Biosystems, Foster City, CA). IFI16 expression was determined with a commercial gene expression assay from Applied Biosystems (Assay-on-Demand: IFI16, Assay No. HS00194261_m1). The housekeeping gene β-actin (Assay-on-Demand: β-actin, Assay No. HS99999903_m1) was used to normalize for variations in cDNA. PCR on each sample was performed

in triplicate in a 25- μ l vol containing 1 μ l diluted cDNA (1:10), 12.5 μ l 2 \times TaqMan universal PCR master mix (Applied Biosystems), and 1.25 μ l 20 \times Assay-on-Demand mix. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were analyzed with a standard curve model.

Western blotting analysis

For crude extract preparation, cells were lysed in 2% sodium dodecyl sulfate (SDS)-lysis buffer containing 50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol (DTT), 2% SDS, and 10% glycerol with the addition of protease and phosphatase inhibitors [0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml pepstatin, 0.1 mM benzamide, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM sodium vanadate, 1 mM sodium fluoride]. Insoluble material was removed by centrifugation at 13,000 rpm for 5 min. Protein concentration was determined by the Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins were separated on 5%, 8.5%, or 10% SDS polyacrylamide gel transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) according to the instruction manual. The membrane was blocked in a blocking solution [10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1% Tween-20, 5% (w/v) low-fat dry milk] overnight at 4°C and incubated with affinity-purified anti-IFI16 rabbit polyclonal antibody, p53, p53 Ser15, actin, and p21. Appropriate secondary antibodies conjugated with horseradish peroxidase conjugate were used (Sigma-Aldrich), and the chemiluminescence reaction was visualized by enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL), according to the manufacturer's instructions. Densitometry was performed by scanning the radiographs and then analyzing the bands with Quantity One software (Bio-Rad Laboratories).

Immunoprecipitation

Cells were washed in cold PBS, harvested, and centrifuged to collect the pellet, which was then incubated for 15 min on ice with a cytoplasmic isolation buffer [10 mM Hepes (pH 7.6), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5% Nonidet-P40 (NP-40), protease inhibitor cocktail (Sigma-Aldrich)]. Following centrifugation, the nuclear pellets were collected by removing the supernatant containing the cytoplasmic extract, washed in cytoplasmic isolation buffer without NP-40, centrifuged, and incubated for 10 min on ice with a nuclear isolation buffer [20 mM Tris-HCl (pH 8.0), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol, protease inhibitor cocktail (Sigma-Aldrich)]. Supernatants containing the nuclear extracts were collected and stored at -70°C. Nuclear extracts (10 μ g proteins) were incubated in a binding buffer [10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 7.5 mM MgCl₂] with antibodies of interest for 50 min at 20°C, and the immune complex was collected with protein G-Sepharose beads (Sigma-Aldrich) for 2 h at 4°C. The beads were washed six times with wash buffer [10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 7.5 mM MgCl₂, 1% NP-40], and the proteins were eluted with Laemmli sample buffer.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [31] with radiolabeled oligonucleotides containing a consensus wtp53 binding site derived from the p21 promoter (wtp53, 5'-GCCGAATTCGAACATGTCCGAACATGTTGAGATCTGCC-3') or a mutp53 binding site (mutp53, 5'-GCCGAATTCGAAAATTCCGAATCCTTTGAGATCTGCC-3'). Briefly, the oligonucleotide probes were labeled with [γ -³²P]adenosine 5'-triphosphate (Amersham, Little Chalfont, UK) and T4 polynucleotide kinase, according to the user's protocol, and finally, column-purified on G-25 Sephadex (Bio-Rad Laboratories). Supershifts were performed using affinity-purified anti-IFI16 rabbit polyclonal antibody or normal rabbit serum as control.

Immunofluorescence

HUVEC, treated for 2 h with increasing H₂O₂ concentrations as indicated, were washed twice with prewarmed Hank's balanced salt solution and incubated with 5 μ mol/L 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 30 min at 37°C. Immunofluorescence was assessed with an Olympus IX70 laser confocal microscope, and images were captured with Fluoview, version 2.0.

RESULTS

IFI16 up-regulation by H₂O₂ occurs in a p53-independent pathway in HUVEC

ROS production following oxidative stress has been recognized as a key chemical event that regulates the signal transduction pathways, which ultimately control gene expression and post-translational modification of proteins [19–22]. To investigate the physiological significance of the IFI16 protein in mediating the response to oxidative stress, its expression was analyzed in proliferating HUVEC treated with low H₂O₂ concentrations. To this end, HUVEC were treated with 10 μ M, 50 μ M, or 250 μ M H₂O₂ concentrations and incubated with DCFH-DA, a redox-sensitive dye that accumulates in the mitochondria and exhibits green fluorescent staining when oxidized to 2',7'-dichlorofluorescein. This technology has been widely used as a sensor for intracellular ROS detection [32]. As shown in **Figure 1A**, HUVEC treated for 2 h with increasing concentrations of H₂O₂ exhibited weak green fluorescent staining at 10 μ M, which increased to a maximum at 50 μ M and 250 μ M concentration. As expected, untreated cells did not display any staining. As 250 μ M H₂O₂ resulted in partial cell cytotoxicity at 24 h after treatment (data not shown), the concentration of 50 μ M was chosen for further investigations. Time-course experiments revealed that upon treatment with 50 μ M H₂O₂, IFI16 started to increase as early as 30 min after treatment, reached a plateau at 6 h, and decreased at 24 h (Fig. 1B). When HUVEC were treated for 6 h with increasing concentrations of H₂O₂, ranging from 1 μ M to 50 μ M (Fig. 1C), IFI16 was barely detectable in untreated cells, increased at 10 μ M, and remained at high levels with 50 μ M. Comparable levels of IFI16 expression were observed upon human IFN- β (1000 IU/ml) treatment. To prove that this pathway is a general response to ROS and not specific to H₂O₂ treatment, HUVEC were treated for 6 h with two other ROS-generating compounds, i.e., SNAP and tBHP [33, 34], and IFI16 expression was analyzed by immunoblotting. As shown in Figure 1D, both compounds induced IFI16 levels comparable with those observed with H₂O₂, demonstrating that IFI16 induction is a general response to ROS.

NAC, a general antioxidant and glutathione precursor that alters the redox state of cells, has been used to investigate the role of ROS in numerous biological and pathological processes [35]. To confirm the ROS capability to trigger IFI16 expression, HUVEC pretreated for 1 h with NAC were incubated for 6 h with H₂O₂ (50 μ M), and the extracts were analyzed for IFI16 expression. As shown in **Figure 2A** (lane 3), pretreatment of endothelial cells with NAC prevented IFI16 accumulation, suggesting a direct role of ROS in IFI16 induction.

Previous reports demonstrated a direct interaction of IFI16 with p53 [11]. However, it is not known whether IFI16 depends on functional p53 for its stimulation, i.e., if it belongs to the growing list of p53-inducible genes. To answer this question, HUVEC were transduced with recombinant retroviruses expressing the E6 and E7 genes of HPV16, known to inactivate p53 and pRb pathways [36]. E6 and E7 gene expression was confirmed by real-time RT-PCR (data not shown). E6/E7-HUVEC were then treated with H₂O₂ (50 μ M) or left untreated,

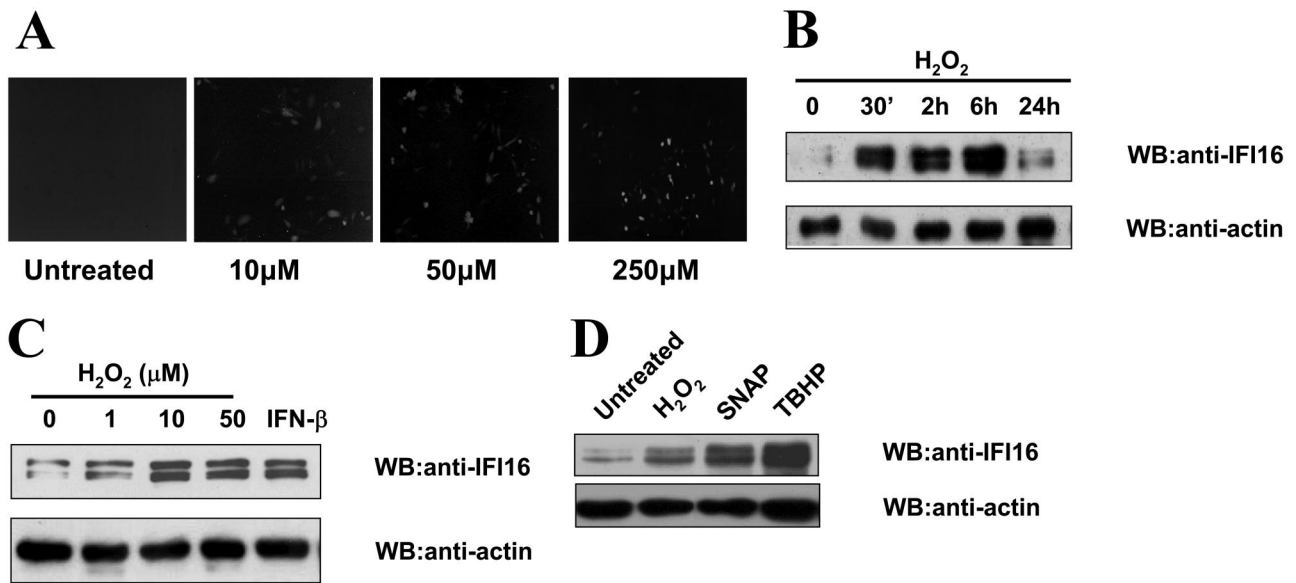


Fig. 1. Effect of H_2O_2 on ROS generation and induction of IFI16 in HUVEC. (A) HUVEC treated with 10, 50, or 250 μM H_2O_2 for 2 h were incubated with DCFH-DA for 30 min at 37°C; cells were washed twice with PBS and analyzed by confocal microscopy. (B) HUVEC ($2.5 \times 10^4/cm^2$) were incubated with 50 μM H_2O_2 for the indicated times. The whole cell lysates were collected and analyzed by Western blotting (WB) using specific antibodies to IFI16 or β -actin as control. (C) HUVEC ($2.5 \times 10^4/cm^2$) were incubated for 18 h with increasing concentrations of H_2O_2 or IFN- β (1000 U/ml). The whole cell lysates were collected and analyzed by Western blotting using specific antibodies to IFI16 or β -actin as control. (D) HUVEC ($2.5 \times 10^4/cm^2$) were incubated with 50 μM H_2O_2 , 0.2 mM tBHP, and 50 μM SNAP for 6 h, and whole cell extracts were analyzed by immunoblotting. Each experiment has been repeated at least three times, and one representative is reported.

and IFI16 expression levels were determined. As shown in Figure 2B, untreated E6/E7-HUVEC displayed IFI16 expression levels comparable with H_2O_2 -treated parental cells, and no further induction was detected after oxidative stress. As expected, p53 protein was undetectable in the extracts from E6/E7-HUVEC, as interaction with HPV16 E6 results in substantially enhanced degradation of p53 [33]. Altogether, these results demonstrate that IFI16 induction by H_2O_2 is independent from functional p53.

A discrete sequence within the 5'-untranslated region of the IFI16 gene, containing an IFN-responsive element, has been shown to be critical for its IFN-induced transcriptional activation [37]. To clarify the mechanisms of IFI16 induction by H_2O_2 , its expression was examined at the mRNA level. To this purpose, HUVEC were stimulated with H_2O_2 (50 μM), and at different time-points, IFI16 mRNA levels were analyzed by real-time RT-PCR. As positive control, HUVEC were exposed to IFN- β (1000 IU/ml). As expected, IFI16 mRNA was up-

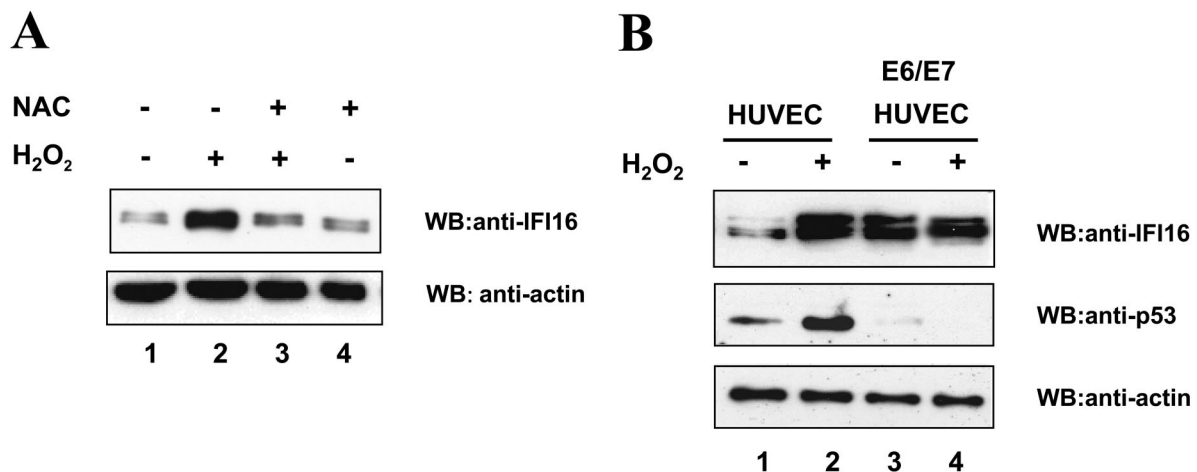
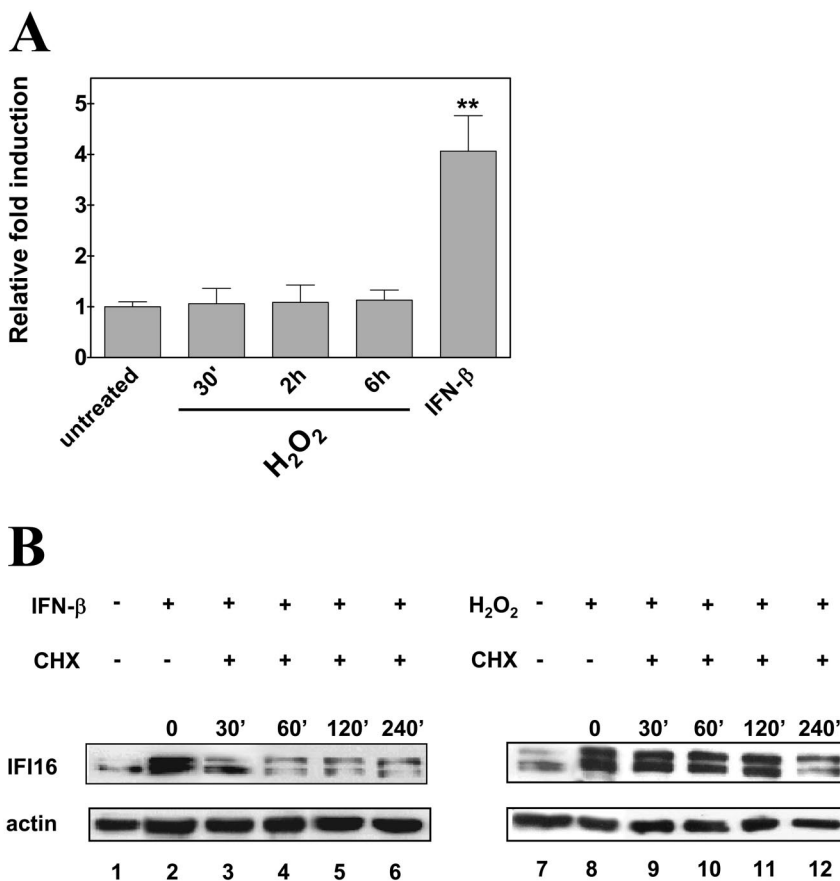


Fig. 2. Effects of NAC or HPV16 E6/E7 oncoproteins on IFI16 induction by H_2O_2 . (A) HUVEC ($2.5 \times 10^4/cm^2$) were treated with 50 μM H_2O_2 for 6 h in the absence or presence of 30 μM NAC. The whole cell lysates were collected and analyzed by Western blotting (WB) using specific antibodies to IFI16 or β -actin as control. (B) HUVEC or E6/E7 HUVEC ($2.5 \times 10^4/cm^2$) were incubated for 6 h with 50 μM H_2O_2 . The whole cell lysates were collected and analyzed by Western blotting using specific antibodies to IFI16, p53, or β -actin as control. Each experiment has been repeated at least three times, and one representative is reported.

Fig. 3. Effect of H₂O₂ on de novo IFI16 mRNA and protein synthesis. (A) HUVEC (2.5×10⁴/cm²) were left untreated or treated for increasing times with 50 μM H₂O₂ or for 6 h with IFN-β (1000 U/ml). Total RNA was extracted, reverse-transcribed, and analyzed by real-time RT-PCR for IFI16 or β-actin expression using commercial gene expression assays from Applied Biosystems. The housekeeping gene β-actin was used to normalize for variations in cDNA. We assigned the value of 1 to the normalized IFI16 level on untreated cells. Each reaction was performed in triplicate, and the histograms represent the mean ± SEM of three independent experiments. (B) HUVEC (2.5×10⁴/cm²) were treated for 6 h with H₂O₂ (50 μM; right panel) or IFN-β (1000 IU/ml; left panel). CHX (120 μg/ml) was added, and cell extracts were harvested at the indicated times. Aliquots (20 μg protein) of whole cell extracts were separated by SDS-PAGE and analyzed by Western blotting. Each experiment has been repeated at least three times, and one representative is reported. **, *P* < 0.01, One-way ANOVA with Bonferroni's multiple comparison test (GraphPad Prism 4.0).



regulated at 6 h after IFN-β treatment (about fourfold) when compared with untreated cells (Fig. 3A). By contrast, H₂O₂ treatment had only a marginal effect on IFI16 mRNA levels (<1.5-fold of induction) at all time-points tested, suggesting that IFI16 accumulation is mainly a result of post-translational mechanisms.

To further confirm that IFI16 induction by H₂O₂ is attributed to an increase in protein stability, we investigated the effect of oxidative stress on its half-life. To this end, cells were treated with H₂O₂ or IFN-β for 6 h, and then CHX (120 μg/ml) was added to inhibit new protein synthesis. Extracts were harvested at the indicated times and separated by SDS-PAGE, and protein half-life was determined by immunoblotting. As shown in Figure 3B (left panel), IFN-β-treated cells exhibited IFI16 half-life of less than 30 min, as the IFI16 protein is barely detectable at 30 min after CHX addition. By contrast, IFI16 half-life in H₂O₂-treated cells was significantly increased, being detectable at 2 h after CHX treatment (Fig. 3B, right panel). Taken together, these results demonstrate that H₂O₂ differentially regulates IFI16 protein induction compared with IFN-β and does not affect its transcriptional activation but rather stabilizes and accumulates the protein at the post-transcriptional level.

In vivo interaction between IFI16 and p53

Previous studies have shown that IFI16 directly binds to the C-terminal region of p53 and augments its transcriptional activation [11, 38]. Cellular stresses, such as oxidative stress, induce p53 phosphorylation in its N-terminal domain and

achieve p53 activation by inhibiting MDM2 binding and nuclear export [24–27]. Based on the finding that H₂O₂ induces IFI16 accumulation, to verify whether this increase corresponded to a gain of function, such as p53 binding and transcriptional activation, reciprocal coimmunoprecipitation experiments were performed. Nuclear extracts from H₂O₂-treated HUVEC were immunoprecipitated with antibodies against IFI16 or p53 and samples immunoblotted with antibodies against p53 or IFI16, respectively. As shown in Figure 4 (lane 4), anti-IFI16 polyclonal antibodies immunoprecipitated endogenous p53, and no bands were observed with the control antibody (lane 3). When the nuclear extracts were immunoprecipitated with anti-p53 and immunoblotted with anti-IFI16 antibodies, a migrating band with a molecular weight corresponding to the B IFI16 isoform was observed (lane 8). By contrast, only barely detectable bands were detected with immunoprecipitates from untreated cells (lanes 2 and 6).

Phosphorylation within or close to the N-terminal domain of p53 is clearly involved in regulation of its stability by interfering with MDM2 binding [24, 25]. To determine whether IFI16 interacts with a phosphorylated form of p53, thus contributing to its stabilization, nuclear extracts from H₂O₂-treated HUVEC were immunoprecipitated with anti-IFI16 antibodies, and samples were immunoblotted with anti-Ser15 p53 antibodies. As shown in Figure 4 (lane 10), p53 bound to IFI16 is indeed phosphorylated within its N-terminal domain at the Ser15 residue. DNase I treatment of H₂O₂-treated HUVEC lysates did not affect immunoprecipitation, suggesting that the

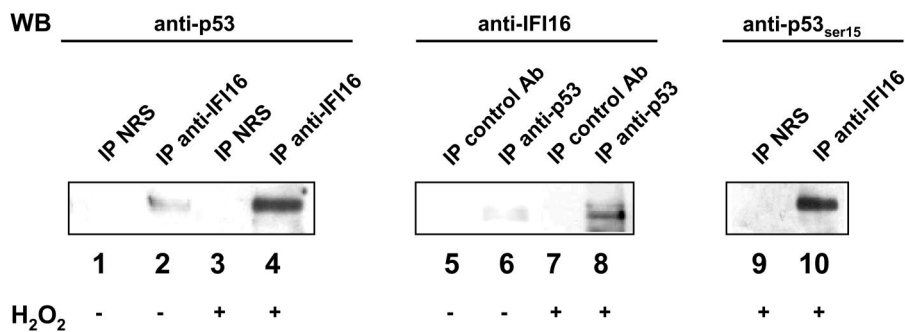


Fig. 4. In vivo interaction of IFI16 with p53 in H_2O_2 -treated HUVEC. Nuclear extracts were prepared from HUVEC ($2 \times 10^4/cm^2$) left untreated (lanes 1, 2, 5, 6) or treated for 6 h with $50 \mu M H_2O_2$ (lanes 3, 4, 7–10), and immunoprecipitations (IP) were performed using antibodies to IFI16 (lanes 2 and 4), p53 (lanes 6 and 8), unrelated mAb (lanes 5 and 7), and normal rabbit serum (NRS; lanes 1 and 3). Bound proteins were separated by SDS-PAGE, and the presence of p53 (lanes 1–4), IFI16 (lanes 5–8), and p53-Ser15 (lanes 9, 10) was detected by Western blotting (WB) with the indicated antibodies. Each experiment was performed twice, and one representative is reported.

proteins reside in the same complex, rather than interact through their DNA (data not shown).

H_2O_2 treatment accumulates IFI16 in the p53-DNA binding complex

We next tested whether upon H_2O_2 treatment, IFI16 and p53 can interact while p53 is bound to DNA. Gel shift assays were performed using double-stranded oligonucleotides derived from the p21 promoter containing wtp53 binding sites and nuclear extracts from HUVEC treated with H_2O_2 ($50 \mu M$). As shown in **Figure 5**, a specific protein DNA complex was observed with labeled wtp53 oligonucleotide that could be competed with a 200-fold excess of cold wtp53 but could not be competed with mutp53 oligonucleotide containing mutations in the p53 binding sequences. As expected, untreated cells contained only barely detectable nuclear p53 activity. Addition of the polyclonal rabbit anti-IFI16 antibodies inhibited specific binding of p53 to DNA (lane 4), and addition of normal rabbit serum had no effect. These findings are consistent with those previously reported by Johnstone et al. [11], showing that addition of anti-IFI16 mAb inhibits binding of p53 to DNA rather than generating supershift of the migrating band.

Effect of IFI16 on p53 transcriptional activity

p53 is a sequence-specific transcription factor that binds to DNA as a tetramer and activates or represses transcription from a large, ever-increasing number of genes [39]. We therefore set out to determine if IFI16 overexpression in HUVEC without H_2O_2 exposure would lead to its binding to phosphorylated p53 and an increase of its transcriptional activity. IFI16 overexpression was achieved by infecting HUVEC with AdVIFI16 for 24 h as shown in **Figure 6A**. As reported earlier, p53 expression levels were also increased as a consequence of IFI16 transduction (Fig. 6A) [15, 38]. Nuclear extracts were immunoprecipitated with anti-FLAG mAb to distinguish exogenous IFI16 or with unrelated mAb as control. The immunoprecipitates from AdVIFI16- and AdVLacZ-infected cells were then immunoblotted with anti-p53 or anti-p53Ser15 antibodies. As shown in Figure 6B, anti-FLAG antibody immunoprecipitated p53, and no bands were observed with the control antibody. Consistent with previous results, p53 bound to IFI16 is phosphorylated on Ser15 and recognized by the specific antibodies.

To evaluate the effect of IFI16 overexpression on p53, a luciferase assay using the p53RELuc or mutp53RELuc re-

porter constructs, containing tandem repeats of the canonical or mutp53 binding site, respectively, was performed. Transiently transfected cells were left untreated or treated with H_2O_2 ($50 \mu M$) or were infected with AdVIFI16 or AdVLacZ recombinant viruses, and luciferase activity was measured 24 h

NRS	-	-	-	+	-	-
anti-IFI16 Ab	-	-	+	-	-	-
wtp53	-	-	-	-	-	+
mutp53	-	-	-	-	+	-
H_2O_2	-	+	+	+	+	+

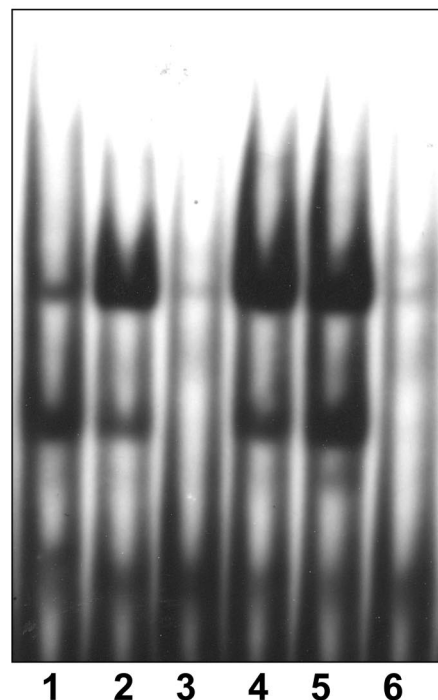


Fig. 5. Effect of H_2O_2 on IFI16 and p53 interaction at the DNA level. Nuclear extracts from HUVEC ($2.5 \times 10^4/cm^2$) treated for 6 h with H_2O_2 ($50 \mu M$) were incubated with ^{32}P -labeled oligonucleotide containing consensus p53 binding sites to form a specific complex. Unlabeled competitor oligonucleotides (200-fold excess) containing wtp53 consensus site or point mutations within the p53 sites that abolished p53 binding (mutp53) were added as indicated by + or -. Supershift experiments were performed with polyclonal rabbit anti-IFI16 antibodies or normal rabbit serum (NRS) used as a control. Each experiment has been repeated three times, and one representative is reported.

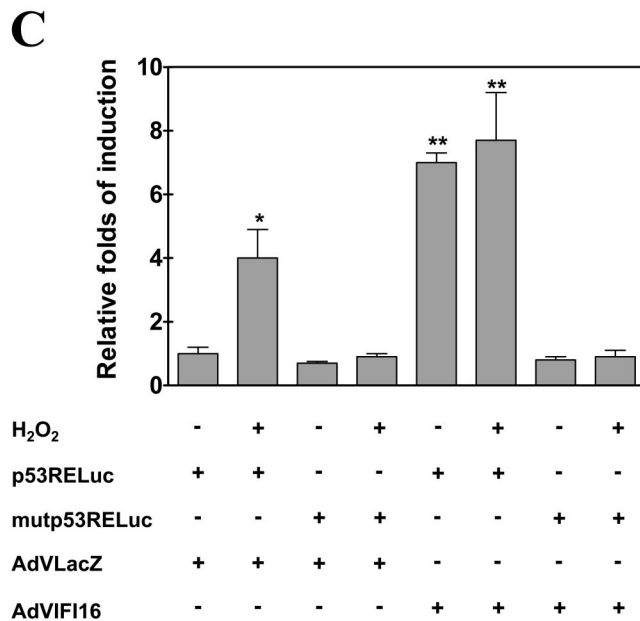
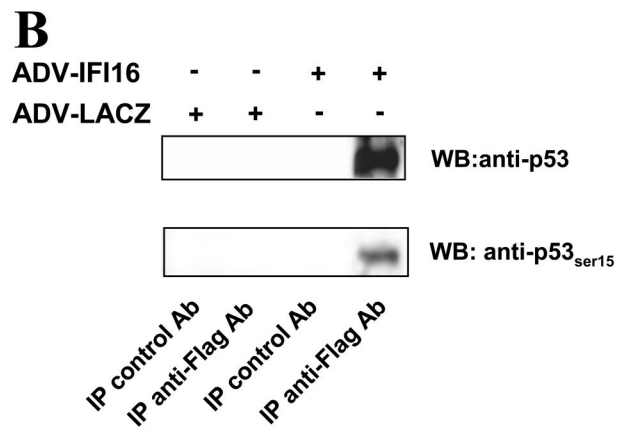
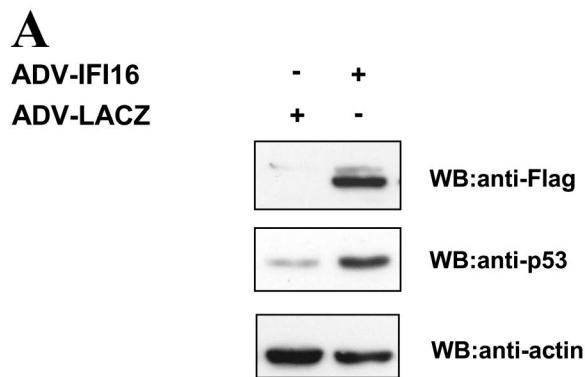


Fig. 6. IFI16 binds phosphorylated p53 in the absence of oxidative stress and triggers transactivation of the p53-responsive sequences. (A) HUVEC ($2 \times 10^4/cm^2$) were infected for 24 h with AdvIFI16 or AdvLacZ (moi 50:1), and nuclear extracts were analyzed by Western blotting (WB) with the indicated antibodies. (B) Nuclear lysates were immunoprecipitated with anti-FLAG mAb or unrelated mAb of the same isotype (control Ab). Bound proteins were separated by SDS-PAGE, and the presence of p53 or p53-Ser15 was detected by Western blotting with the indicated antibodies. (C) p53RELuc or mutp53RELuc reporter constructs, containing tandem repeats of the canonical or mutp53 binding site, respectively, upstream of the luciferase gene, were transfected into HUVEC ($2.5 \times 10^4/cm^2$). Twenty-four hours later, cells were left untreated, treated with H₂O₂ (50 μ M), or infected with AdvIFI16 or AdvLacZ (moi 50:1). After an additional 24 h, protein extracts were assessed for luciferase activity. The resulting luciferase activity is expressed as fold induction relative to basal levels measured in cells transfected with p53RELuc and infected with AdvLacZ, which was set at 1. The data shown are the values (\pm SEM) from three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$, One-way ANOVA with Bonferroni's multiple comparison test.

later. As shown in Figure 6C, HUVEC, transfected with the p53RELuc construct, infected with AdvLacZ, and treated with H₂O₂, displayed fourfold activation compared with untreated HUVEC transfected with the same construct, demonstrating that ROS-dependent IFI16 accumulation leads to transactivation of the p53-responsive sequences. By contrast, IFI16 overexpression generated higher levels of luciferase activity (>sevenfold) independently from H₂O₂ treatment, demonstrating that IFI16, per se, is sufficient to stimulate the reporter activity driven by the p53-responsive sequence and does not require other signals provided by oxidative stress. That this transactivation is mediated by p53 is further confirmed by the finding that AdvIFI16 was unable to induce any increase in enzymatic activity when HUVEC cells were transfected with the mutp53RELuc reporter construct bearing the mutp53 binding site.

To assess the physiological response of the cell to the activation of the p53-responsive elements upon sustained IFI16 expression, the mRNA and protein levels of a downstream p53 gene, i.e., p21, were evaluated. HUVEC were infected with AdvIFI16 or AdvLacZ, and p21 mRNA and protein levels were measured by real-time RT-PCR or immunoblotting, respectively. Consistent with the results obtained in

transfection experiments, sustained expression of IFI16 produced an increase in p21 mRNA (Fig. 7A) and p21 protein (Fig. 7B), suggesting that IFI16 is sufficient, per se, to activate p53 transcriptional activity and expression of downstream genes.

DISCUSSION

Oxidative stress resulting from an excessive generation of ROS has been implicated as an important mechanism that modulates various signaling pathways and contributes to endothelial dysfunction [17–20]. To defend themselves against DNA oxidation induced by ROS, proliferating cells arrest their cell cycle to prevent the mutated DNA from being replicated and transferred to daughter cells. Formation of ROS is not always associated with cell damage but is also induced during many physiologic, cellular processes, including the regulation of signal transduction, gene expression, and proliferation [20–22]. Therefore, depending on the concentration, the molecular species, and the subcellular localization, ROS exerts two physiopathological effects: damage to various cell components and activation of specific signaling pathways.

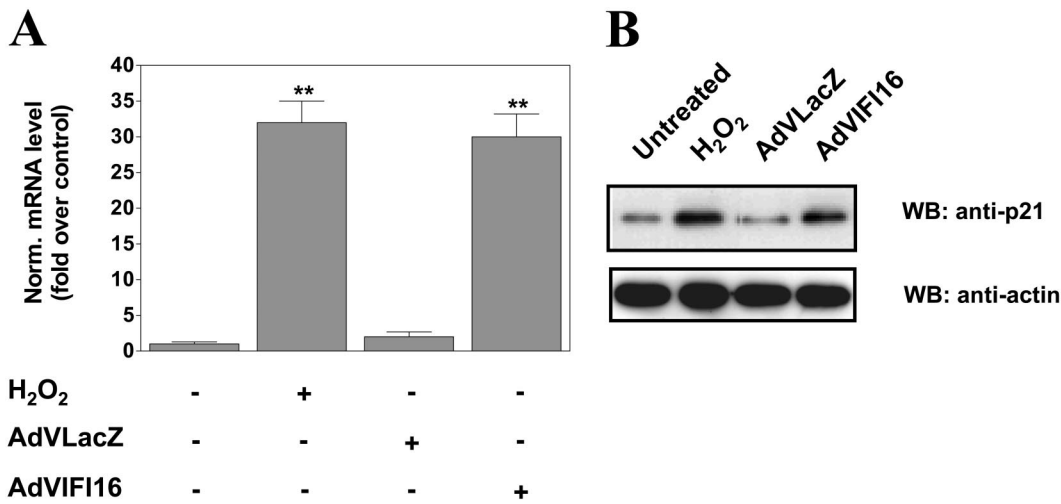


Fig. 7. IFI16 overexpression stimulates an increase of p21 mRNA and protein levels. (A) HUVEC ($2.5 \times 10^4/\text{cm}^2$) were left untreated, treated with $50 \mu\text{M}$ H₂O₂, or infected with AdVIFI16 or AdVLacZ (moi 50:1) for 24 h. Total RNA was extracted, reverse-transcribed, and analyzed by real-time RT-PCR for p21 or β -actin mRNA expression using commercial gene expression assays from Applied Biosystems. The housekeeping gene β -actin was used to normalize for variations in cDNA. We assigned the value of 1 to the normalized p21 level on untreated and uninfected cells. (B) HUVEC ($2.5 \times 10^4/\text{cm}^2$) were left untreated, treated with $50 \mu\text{M}$ H₂O₂, or infected with AdVIFI16 or AdVLacZ (moi 50:1) for 24 h. The whole cell lysates were collected and analyzed by Western blotting (WB) using specific antibodies to p21 or β -actin as control. Each experiment has been repeated three times, and one representative is reported. **, $P < 0.01$, One-way ANOVA with Bonferroni's multiple comparison test.

Immunohistochemical analysis performed in our [14] and other laboratories [15] demonstrated that the human IFI16 gene, in addition to hematopoietic tissues, is highly expressed in endothelial cells. By using a reliable HSV-derived replication defective vector to efficiently transduce IFI16 into primary HUVEC, we have previously observed that its sustained expression inhibited tube morphogenesis and proliferation, accompanied by significant induction of p53, p21, and hypophosphorylated Rb [16].

In this study, the physiological significance of the IFN-inducible gene IFI16 in mediating the cellular response to oxidative stress induced by sublethal H₂O₂ concentrations was examined. We found that H₂O₂ produced rapid accumulation of IFI16. This was not dependent on functional p53 and pRb proteins, as similar induction was observed in HPV16 E6/E7-immortalized HUVEC. Regulation of IFI16 accumulation by H₂O₂ appears to be the result of its redox activity, as two other compounds, i.e., SNAP and tBHP, known to generate ROS intracellularly [33, 34], caused an IFI16 increase as well. Moreover, this increase was inhibited by addition of NAC, a source of sulfhydryl groups in cells and scavenger of free radicals as it interacts with ROS, such as OH[•] and H₂O₂ [35].

The activity of IFI16 could be regulated at the transcriptional level. However, the results obtained demonstrate that following oxidative stress caused by low H₂O₂ concentrations, IFI16 induction is a consequence of protein accumulation rather than transcriptional activation. This hypothesis rises from the observation that IFI16 mRNA, evaluated by real-time RT-PCR, displayed a modest increase compared with that from untreated HUVEC. By contrast, but consistent with the results previously reported by Clarke et al. [37], a significant increase in transcriptional activation of the IFI16 promoter and its mRNA level was observed following IFN treatment. Further support to these conclusions came from the finding that inhi-

bition of the novo protein synthesis achieved by CHX addition did not affect the levels of IFI16 protein and down-regulating IFI16 induction by IFN- β . Thus, low H₂O₂ concentrations sufficient to induce intracellular ROS accumulation and IFNs exploit different mechanisms to accumulate IFI16 protein.

A number of DNA-damaging treatments, including H₂O₂, hypoxia, and depletion of ribonucleotide pools, increase the level of p53 by post-transcriptional regulation [38]. Moreover, it has been demonstrated that IFI16 directly binds to the C-terminal region of p53 and augments p53-mediated transcriptional activation [11]. Our studies now extend the role of IFI16 by showing that it is also involved in p53 transcriptional activation by oxidative stress. These findings demonstrate a possible link between gene induction following H₂O₂ stimulation and p53-mediated cellular events. Although the physiological significance of the IFI16-p53 interaction is still under investigation, the finding that IFI16 binds p53 phosphorylated at its N terminus upon H₂O₂ treatment suggests that it bona fide contributes to its stabilization and transcriptional activation. ROS has been implicated in DNA damage by genotoxic agents such as UV and ionizing radiation or oxidative stress. Subsequent accumulation of ROS-damaged DNA is a critical event during carcinogenesis and aging [40]. Cellular stresses induce p53 phosphorylation in its N-terminal domain [24–26]. Several mechanisms for stress-induced p53 phosphorylation have been identified including DNA-dependent protein kinase [41], ataxia teleangiectasia mutated (ATM) kinase [42], and the Ataxia Teleangiectasia and Rad-3-related kinase [43]. These kinases represent a subgroup of the phosphatidylinositol kinase superfamily that broadly functions in response to DNA damage or control of the cell cycle. IFI16 contains four potential phosphorylation consensus motifs (S/TQ) for ATM kinase, namely at positions 153, 239, 241, and 628.

The finding that anti-IFI16 antibodies immunoprecipitate from the lysates of H₂O₂-treated HUVEC the ATM kinase in addition to p53 (data not shown) suggests that IFI16 may help to stabilize p53 by recruiting this kinase into the complex. Our results are to some extent consistent with those reported by Xin et al. [44], showing that ectopic expression of IFI16 in human prostate cancer cell lines leads to up-regulation of p21 and contributes to senescence-associated, irreversible growth arrest. IFI16 overexpression in endothelial cells does not lead to senescence or apoptosis but to growth arrest in G1 and G2/M phases accompanied by an increase of p21 [16]. On the contrary, Kwak et al. [45] reported that the inhibition of endogenous IFI16 expression by small, interfering RNA in a tumor cell line, U2OS, expressing high levels of endogenous IFI16, induces p21^{WAF1} mRNA and protein expression through p53 and results in cell-cycle arrest. In subsequent studies, however, the same investigators [38] demonstrated that overexpression of IFI16 in the MCF7 carcinoma cell line enhanced p53 transcriptional activity accompanied by p21^{Waf1} activation in cells exposed to ionizing radiation. These controversial results clearly indicate that IFI16 activity may dramatically change depending on the cell type and experimental system.

In summary, the data presented here demonstrate that oxidative stress leads to accumulation of the IFN-inducible protein IFI16, binding to phosphorylated p53 on Ser-15, and transcription of the p53 target gene p21^{WAF1}. These data suggest a novel role for IFI16 in the response of endothelial cells to oxidative stress.

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