Stabilization of p53 Is a Novel Mechanism for Proapoptotic Function of NF-κB*

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‡ The abbreviations used are: NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PUMA, p53 up-regulated modulator of apoptosis; EMSA, electrophoretic mobility shift assay; NAC, N-acetylcysteine; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate; ROS, reactive oxygen species.

Both pro- and antiapoptotic activities of NF-κB transcription factor have been observed; however, less is known about the mechanism by which NF-κB induces apoptosis. To elucidate how NF-κB regulates proapoptotic signaling, we performed functional analyses using wild-type, ikk1−/−, ikk2−/−, rela−/− murine fibroblasts, MDAPanc-28/Puro, MDAPanc-28/IκBoM, and HCT116/p53+/− and HCT116/p53−/− cells with investigational anticancer agent doxycycline as a superoxide inducer for generating apoptotic stimulus. In this report, we show that doxycycline increased superoxide generation and subsequently activated NF-κB, which in turn up-regulated p53 expression and increased the stability and DNA binding activity of p53. Consequently, NF-κB-dependent p53 activity induced the expression of p53-regulated genes PUMA and p21 as well as apoptosis. Importantly, lack of RelA, IKK, and p53 as well as expression of a dominant negative IκBo (IκBoM) inhibited NF-κB-dependent p53 activation and apoptosis. The doxycycline-induced NF-κB activation was not inhibited in HCT116/p53−/− cells. Our results demonstrate that NF-κB plays an essential role in activation of wild-type p53 tumor suppressor to initiate proapoptotic signaling in response to overgeneration of superoxide. Thus, these findings reveal a mechanism of NF-κB-regulated proapoptotic signaling.

The mammalian Rel/NF-κB family, which consists of RelA (p65), Rel (v-rel), RelB, p50 (p105), and p52 (p100), plays a key role in the regulation of immune response, inflammatory reactions, cell proliferation, and apoptosis (1–4). NF-κB is activated through a complex network of kinase signaling cascades in response to various stimuli (5–8). Recently we have demonstrated the mechanism by which pro-inflammatory cytokines induce biphasic NF-κB activation (9). IKK1 and IKK2 are the essential kinases in signal-induced phosphorylation of IκB proteins and subsequent activation of NF-κB, which in turn induces a large number of genes to affect the subsequent response and phenotype of a cell (5, 10–13). RelA, the p65 subunit of Rel/NF-κB transcription factors has been shown to play a key role in protecting cells from proapoptotic stimuli (14, 15). Similarly, radiation-, daunorubicin-, or TNF-α-induced apoptosis is potentiated in HT1080 human fibrosarcoma and Jurkat cell lines transfected with dominant negative IκBo (16, 17).

Many studies have shown that proapoptotic signals can induce Rel/NF-κB, which in turn induces expression of the genes involved in suppressing apoptotic signals (4) (18). Inhibitors of apoptosis c-IAP1 and c-IAP2 and Bcl-2 family members Bcl-xL and Bfl1/A1 have been proposed to mediate NF-κB-dependent antiapoptotic signaling (19–23). Conversely, a proapoptotic aspect of RelA activity has also been reported (24–28). For example, the induction of apoptosis by glucocorticoids is promoted by inhibition of NF-κB, whereas apoptosis induced in the same cells by stimulation with phorbol ester and ionomycin for mimicking T-cell activation requires NF-κB (28). It has also been shown that NF-κB induces cell death following T-cell receptor engagement or DNA-damaging agents (24–26).

Other reports have shown that NF-κB activation is required for the onset of apoptosis induced by alpavirus or kainic acid (27, 29). These studies further emphasize that the function of NF-κB can be proapoptotic or antiapoptotic, depending on cell type, extent of NF-κB activation, and nature of the apoptotic signals. However, how NF-κB induces apoptosis and which proapoptotic downstream target genes is induced by NF-κB still remains unclear.

The tumor suppressor p53 plays an important role in regulating expression of genes that mediate cell cycle arrest and/or apoptosis in response to genotoxic insults (30, 31). Reactive oxygen species (ROS) are some of the potent activators of p53 and they appear to be key factors generated in chemotherapeutic agents induced p53 activation (32). The important function of p53 in mediating hydrogen peroxide-induced apoptosis is demonstrated in p53-null cells, suggesting that loss of the function of p53 is a contributing factor to the chemotherapeutic resistance in tumors (33, 34). Following environmental insults, p53 is activated by post-translational modifications such as phosphorylation and acetylation that increase its protein stability and enhance its DNA binding activity (35–37). However,
Induction of p53-dependent Apoptosis by NF-κB

It is unclear how these posttranslational mechanisms that modulate p53 activity are regulated by ROS. Activated p53 up-regulates expression of several of its downstream target genes, including p53 upregulated modulator of apoptosis (PUMA) (38, 39) and cell cycle regulator p21WAF1 (40, 41), and thus the high levels of p53 activity can either result in cell cycle arrest or directly promote cell death (42, 43). A number of factors including the cell type, the specific insults, and the extent of the damage may contribute this decision for apoptosis or cell cycle arrest. In response to many inducing agents, the activity of a well-documented antia apoptotic transcriptional factor, NF-κB, and proapoptotic factor, p53, are simultaneously activated (44–46). The functional NF-κB and p53 activity may modulate each other, which in turn would affect the subsequent responses (47, 48).

Doxycycline and the newly developed chemically modified tetracycline derivatives COL-3 have been evaluated in preclinical cancer models and early clinical trials because these agents inhibit various zinc-dependent enzymes of the matrix metalloproteinase family and induce apoptosis in a number of cancer cell lines (49–52). However, the mechanism by which doxycycline induces apoptosis remains unclear. Kroon and co-workers (53, 54) showed that tetracycline acts as an anticancer agent by preferentially inhibiting mitochondrial protein synthesis, including cytochrome c oxidase, the key components of electron transport chain, and decrease of its synthesis may lead to a disruption of electron transport function and lead to electron leakage from the respiratory chain to O2, thus resulting elevated levels of superoxide radicals.

In this study, we performed functional analyses using ikk1−/−, ikk2−/−, and rela−/− fibroblasts and MDAPan-28/IkBaM and HCT116/p53−/− cells to decode the role of NF-κB in regulating p53-dependent proapoptotic signaling in response to doxycycline-induced ROS. Our study reveals a mechanism by which NF-κB functions as a proapoptotic factor by activating the p53 signaling pathway for initiating cell apoptosis.

EXPERIMENTAL PROCEDURES

Reagents—Doxycycline was purchased from Sigma. Oligodeoxyribonucleotides were purchased from Sigma-Genosys. A stock solution of 8 mg/ml doxycycline was prepared immediately before its use in phosphate-buffered saline (PBS) with a pH of 7.4. Stock solutions were diluted in PBS to allow a 1% volume addition of diluted solutions to the experimental wells. Control wells were added with 1% volume with PBS. Primary antibodies for immunoblotting (IkBa, IkBα, and β-actin) were purchased from Santa Cruz Biotechnology, Inc. Antibodies for p53 immunoblotting and EMSA were purchased from Calbiochem. The proteasome inhibitor FS-341 was reconstituted in PBS and used in 100 mM final concentrations. Radiotisopotes were purchased from Amersham Biosciences.

Cell Culture—The human pancreatic tumor cell line MDAPan-28, which was originally established by Frazier et al. (55), and HCT116 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-incubated fetal bovine serum. The wild-type, IkBaM, and IkBaE, and rela−/− fibroblasts and MDAPan-28/IkBaM and HCT116/p53−/− cells were the cell lines (49, 50). Briefly, end-labeled DNA probes (wild-type B: 5′-AGTGAAGGGAGTGTCAGGC-3′, mutant B: 5′-AGTGAAGGGAGTGTCAGGC-3′, p53: 5′-GTCAGGACATGTCCCAACATGTTGACTG-3′), Sp-1: 5′-ATTCT-AGTCGCTGGGCGCGCCGAC-3′, and Oct-1: 5′-TGTGAAATGCTACTGAACTGAAA-3′ were chosen to label Hu and UV cross-linked. The blots were hybridized with human cDNA probes labeled with [32P]dCTP (56). DNA was purified from cells by phenol/chloroform and chloroform. DNA fragmentation in apoptotic cells was determined by gel electrophoresis. The cells (50 × 10⁶) treated with doxycycline for the specified times were collected and washed with PBS, and nucleic acid was extracted with proteinase K digestion buffer (100 mg/ml ribonuclease A, 0.5% SDS, and 20 μg/ml of RNase) at 37 °C for 1 h. Then, 100 μg/ml of proteinase K was added, and the sample was incubated at 50 °C for 3 h. DNA was extracted with phenol/chloroform and chloroform. The aqueous phase was precipitated with two volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate for 30 min on ice. The DNA pellet was then washed with 70% ethanol and resuspended in 50 μl of Tris-EDTA buffer. The absorbance of the DNA solution at 260 and 280 nm was determined by spectrophotometry. The extracted DNA (40 μg/lane) was subjected to electrophoresis on 2% agarose gels. The gels were stained with ethidium bromide and then photographed.

Northern Blot Analysis and RT-PCR—For Northern blot analysis, total RNA was examined using the TRizol reagent (Invitrogen). Fifteen micrograms of RNA was electrophoresed on a 1% denaturing formaldehyde agarose gel, transferred to a nylon membrane in the presence of 20× SSC, and UV cross-linked. The blots were hybridized with human cDNA probes labeled with [32P]dCTP by random labeling kit (Roche Applied Science). Equal loading of mRNA samples was monitored by hybridizing the same membrane filter with the cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously (61). Semi-quantitative RT-PCR analysis was performed using a pair of PUMA primers (5′-GCAGCCGACATGTTGACTG-3′ and 5′-CATGTGTCAGGAGATGCCC) and separated on an 8% polyacrylamide gel.

Western Blot Analysis—Cytoplasmic extracts were prepared as described previously (59, 60) and were separated on 10% SDS-PAGE by electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were incubated in 5% nonfat milk in PBS containing 0.2% Tween-20 and incubated with affinity-purified monoclonal antibodies against p53 (Calbiochem); Ser20-phosphorylated p53, which recognizes only Ser20 phosphorylated p53 (New England Biolabs); β-actin (M2 Sigma); and rabbit

At room temperature for 1 h, and DNA content was determined by flow cytometry using a FACScan flow cytometer (BD Biosciences).
polyclonal antibodies against 1xB, and 1xBp (Santa Cruz Biotechnology). The membranes were washed in PBS containing 0.2% Tween-20 and probed with horseradish peroxidase-coupled secondary goat anti-rabbit or anti-mouse IgG antibodies (Amersham Biosciences). The Lumi-Light Western blot substrate (Roche Applied Science) was used for detection. For determining half-life for p53, MDAPanc-28/Puro, or MDAPanc-28/1xBoM cells were treated with 50 μg/ml of doxycycline for 24 h, followed by cycloheximide (10 μg/ml) addition. The cytoplasmic and nuclear protein extracts were isolated 2, 4, and 8 h after addition of cycloheximide. p53 levels were examined by Western blot analysis with the monoclonal antibody against p53 (Calbiochem).

Metabolic Labeling and Immunoprecipitation—Pulse chase experiments with [35S]methionine labeling were carried out by culturing the cells in fresh medium including 10% dialyzed fetal calf serum for 1 day, followed by adding 300 μCi of L-[35S]methionine (Amersham Biosciences) in 8 ml of methionine-free medium for 24 h with simultaneous stimulation either with PBS or 50 μg/ml of doxycycline, washing with PBS twice, and further incubating in a medium containing 10% fetal calf serum and 10 mM non-labeled methionine for the indicated time. To equilibrate detection of p53, 200 and 25 μg of crude cell extracts from PBS- and doxycycline-treated cells were used for p53 immunoprecipitation, respectively, and separated by SDS-PAGE, and visualized by phosphorimaging and autoradiography.

RESULTS

NF-κB Activation Induced p53 Activity in Response to Doxycycline Stimulation—To provide a better understanding of the molecular basis of doxycycline-induced cytotoxicity in cancer cells, we first determined the effect of doxycycline on NF-κB activation using MDAPanc-28/Puro and MDAPanc-28/1xBoM cells. MDAPanc-28/1xBoM human pancreatic tumor cells were generated by pooling puromycin-resistant cells after infection with a retrovirus with or without expression of a FLAG-tagged, generated by pooling puromycin-resistant cells after infection with a retrovirus with or without expression of a FLAG-tagged, generated by pooling puromycin-resistant cells after infection with a retrovirus with or without expression of a FLAG-tagged.

Northern blot analysis to compare the expression of p21WAF1/CIP1 and PUMA in doxycycline-stimulated MDAPanc-28/Puro and MDAPanc-28/1xBoM cells. The results showed that the expression of both p21WAF1/CIP1 and PUMA was induced after doxycycline stimulation (Fig. 3, A and B). The basal and doxycycline-induced p21WAF1/CIP1 mRNA levels and doxycycline-induced PUMA expression were almost completely inhibited in MDAPanc-28/IxBoM cells (Fig. 3, A and B, lanes 4–6). These results demonstrate that doxycycline induces p21WAF1/CIP1 and PUMA expression and suggest that doxycycline-induced expression of these p53 target genes is NF-κB-dependent.

Since doxycycline-mediated induction of p53 activity and p53 downstream target gene expression depends on NF-κB activity, we sought to determine whether inhibition of NF-κB activity augmented or suppressed doxycycline-mediated cell cycle arrest or apoptosis. First, cell cycle profiles of these cells were assayed by flow cytometry analysis. Results show that no significant difference was observed between unstimulated MDAPanc-28/Puro and MDAPanc-28/IxBoM cells and doxycycline-induced NF-κB activity plays a role in inducing G1/S arrest in doxycycline-treated MDAPanc-28/Puro cells (Fig. 3C). We also found that doxycycline stimulation significantly reduced the number of viable MDAPanc-28/Puro cells, but not of viable MDAPanc-28/IxBoM cells, suggesting that NF-κB activity may be required for doxycycline-mediated cytotoxicity (Fig. 3D).

To determine whether doxycycline-mediated cytotoxicity is caused by induction of apoptosis in these cells, a DNA fragmentation assay was performed by agarose gel electrophoresis. As shown in Fig. 3E, DNA fragmentation occurred from 48 to 96 h after doxycycline stimulation in MDAPanc-28/Puro cells (Fig. 3E, lanes 3–5). Interestingly, DNA fragmentation was detected only at 96 h after doxycycline stimulation in MDAPanc-28/IxBoM cells (Fig. 3E, lane 11). These results suggest that doxycycline-induced DNA fragmentation was delayed by overexpression of IxBoM, and that NF-κB activity is involved to initiate proapoptotic signaling in doxycycline stimulation.

p53 Activation by NF-κB Is Involved in Doxycycline-Mediated Apoptosis—To determine whether p53 activity is required for doxycycline-induced apoptosis, we carried out our analyses using HCT116p53−/− and HCT116p53−/− cells (57). Fig. 4, A and B show that the expression of p53 mRNA and protein was only detected in HCT116p53−/− cells. Doxycycline stimulation sub-
Fig. 1. Doxycycline induces NF-κB activation and subsequent p53 activity. A, expression of IκBoM. Expression of IκBoM was determined by Western blotting using cytoplasmic extracts from pooled puromycin-resistant control MDAPanc-28 (MDAPanc-28/Puro, Puro) and MDAPanc-28 cells expressing IκBoM (MDAPanc-28/IκBoM, IκBoM) with anti-IκBo antibody. Endogenous IκBo and IκBoM are indicated. Equal loading was monitored by reprobing the filter with an anti-β-actin antibody. B, IκBoM inhibits constitutive NF-κB activity. Nuclear extracts prepared from MDAPanc-28/Puro and MDAPanc-28/IκBoM cells were subjected to EMSA with an NF-κB probe. An Oct-1 probe was used as a control for quality and quantity of cell extracts. C, IκBoM inhibits TNF-α-induced NF-κB activation. MDAPanc-28/Puro and MDAPanc-28/IκBoM cells were stimulated with 10 ng/ml of TNF-α for the times indicated. After stimulation, nuclear protein extracts were isolated and subjected to NF-κB EMSA analysis. An Oct-1 probe was used as the control. D, dose-dependent induction of NF-κB and p53 by doxycycline. Nuclear and whole cell extracts prepared from MDAPanc-28/Puro (Puro) cells stimulated with doxycycline for the indicated dose were subjected to NF-κB EMSA with an Oct-1 probe as the control and p53 Western blot analysis with an β-actin as the loading control. E, doxycycline activates NF-κB. Nuclear extracts prepared from MDAPanc-28/Puro (Puro) and MDAPanc-28/IκBoM (IκBoM) cells stimulated with 50 μg/ml of doxycycline for the indicated times were subjected to NF-κB EMSA. An Oct-1 probe was used as the control. NS, nonspecific bands; FP, free probe. F, doxycycline induces IκBo protein degradation. The cytoplasmic extracts prepared from E were subjected to Western blot analysis using anti-IκBo polyclonal antibodies as indicated. The membrane was reprobed for expression of β-actin as a loading control. G, doxycycline-induced p53 DNA binding activity requires NF-κB activation. Nuclear extracts used in E also were analyzed by EMSA with a p53 probe corresponding to the p53-binding site of the human p21\(^{\text{waf1}}\) promoter. An Oct-1 probe was used as a loading control for the nuclear extracts, and doxycycline-induced p53 DNA binding activity is indicated. H, specificity of the p53 DNA binding activity in MDAPanc-28 cells was characterized. Competition and supershift analyses were carried out with a 50× excess of unlabeled p53 probe (lane 2), Sp-1 probe (lane 3), and anti-p53 antibody (lane 4). p53 DNA binding activity and the supershifted band (SSI) are indicated.
MDAPanc-28/Puro cells exhibited a time-dependent increment in the p53 mRNA level (Fig. 6A). In contrast, the basal level of p53 mRNA expression was reduced and doxycycline-induced p53 expression was attenuated in MDAPanc-28/IκBαM cells (Fig. 6A, lanes 4–6). These results suggest that the doxycycline-induced expression of p53 mRNA may be in part NF-κB-dependent. Western blot analysis performed with nuclear extracts using an anti-p53 monoclonal antibody demonstrated that a large increase in the level of p53 protein was observed following doxycycline stimulation of MDAPanc-28/Puro cells (Fig. 6B, lanes 1–3). In contrast, the level of p53 protein was undetectable in MDAPanc-28/IκBαM cells (Fig. 6B, lanes 4–6). Since the increase in the level of p53 mRNA alone, as shown in Fig. 5A, cannot explain the doxycycline-induced high levels of p53 protein in MDAPanc-28/Puro cells, suggesting that additional regulatory steps such as post-translational stabilization of p53 protein are possibly involved in doxycycline-induced p53 activation.

To examine the stability of p53 protein in doxycycline-stimulated MDAPanc-28/Puro cells, we directly measured its half-life in MDAPanc-28/Puro and MDAPanc-28/IκBαM cells. The half-life of p53 protein in MDAPanc-28/Puro cells was about 20 min, whereas in doxycycline-stimulated MDAPanc-28/Puro cells, it was much longer (Fig. 6C, lane 9). The level of p53 protein was barely detectable in MDAPanc-28/IκBαM cells (Fig. 6C, lanes 4–6), even though 8 times as much protein extracts (200 μg/ml) from unstimulated cells were loaded (Fig. 6C, lanes 1–6). To further analyze the stability of p53 protein in doxycycline-stimulated MDAPanc-28 cells, we directly measured its half-life in the presence of the protein synthesis inhibitor cycloheximide. The stability of p53 protein was greatly increased and its half-life in doxycycline-stimulated MDAPanc-28 cells is more than 8 h (Fig. 6D, lanes 9–12 and Fig. 6F). No change in the level of p53 protein was detected in the extracts from MDAPanc-28/IκBαM cells with or without doxycycline stimulation (To aid the detection of the extremely low level of p53 protein, 4 times as much protein extracts were loaded to gels) (Fig. 6E, lanes 5–8 and lanes 13–16). Thus, our results showed that doxycycline-induced p53 expression and increased p53 stability are NF-κB-dependent.

NF-κB-dependent Reduction in the Level of Hdm2 Proteins May Be Involved in Stabilization of p53—To identify a possible mechanism for p53 stabilization, we examined the level of Hdm2 protein from MDAPanc-28/Puro and MDAPanc-28/IκBαM cells stimulated with doxycycline at different time points. Fig. 7A shows the locations of the epitopes for the anti-HDM2 monoclonal antibodies used in the immunoblotting.
Fig. 3. Doxycycline-inducible expression of p53 downstream target genes p21<sup>vasf</sup> and PUMA is NF-κB-dependent. A, induction of p21<sup>vasf</sup> by doxycycline is dependent on NF-κB activity. RNA was isolated from MDAPanc-28/Puro (lanes 1–3) and MDAPanc-28/IxBoM (lanes 4–6) cells stimulated with 50 μg/ml of doxycycline for the indicated times and analyzed by Northern blotting with a human p21<sup>vasf</sup> cDNA probe; the same blot was washed and rehybridized to a gapdh cDNA probe. B, induction of PUMA by doxycycline is also dependent on NF-κB activity. Northern blot analyses were carried out to determine PUMA expression in the doxycycline-stimulated MDAPanc-28/Puro (lanes 1–3) and MDAPanc-28/IxBoM (lanes 4–6) cells using the same RNA from A. C, doxycycline-induced cell cycle arrest is NF-κB-dependent. MDAPanc-28/Puro and MDAPanc-28/IxBoM cells were incubated with 50 μg/ml of doxycycline for the indicated times and were collected for fluorescent-activated cell sorting analysis. D, IxBoM inhibits doxycycline-mediated cytotoxicity. MDAPanc-28/Puro (Puro) and MDAPanc-28/IxBoM (IxBoM) cells were incubated with 50 μg/ml of doxycycline. At each indicated interval, cells were collected and their survival was determined as described under “Experimental Procedures.” E, doxycycline-induced apoptosis is dependent on NF-κB activity. MDAPanc-28/Puro (lanes 1–5) and MDAPanc-28/IxBoM (lanes 7–11) cells were treated with 50 μg/ml of doxycycline for the indicated times, and DNA fragmentation assays were performed. Lane 12 is a DNA marker.

Doxycycline stimulation greatly reduced the level of 90 kDa Hdm2 protein in MDAPanc-28/Puro cells and at 48 h of doxycycline stimulation Hdm2 protein was barely detectable (Fig. 7, B and C). Interestingly, the level of a 55 kDa Hdm2 protein, known to be identified by anti-HDM2 antibody (M7815), increased by doxycycline stimulation as the 90 kDa Hdm2 protein decreased in doxycycline-stimulated MDAPanc-28/Puro cells (Fig. 7B). The 55 kDa Hdm2 protein was not detected by anti-HDM2 antibody (M7815) in doxycycline-stimulated MDAPanc-28/IxBoM cells (Fig. 7B). Another anti-HDM2 antibody (M4308), which recognizes the N terminus of Hdm2 protein, did not detect the 55 kDa Hdm2 protein in both doxycycline-stimulated MDAPanc-28/Puro and MDAPanc-28/IxBoM cells (Fig. 7C). These results suggested that NF-κB-dependent p53 stabilization involves the reduction of the level of 90 kDa Hdm2 protein, a key p53 regulator, which functions as an ubiquitin E3 ligase to promote p53 degradation.

Because phosphorylation of p53 is a major post-translational modification that increases p53 stability and activity, we determined whether phosphorylation of p53 is induced by doxycycline. As shown in Fig. 8A, p53 phosphorylation at Ser<sup>20</sup> was induced by doxycycline stimulation in MDAPanc-28/Puro cells. IxBoM degradation, a key step in NF-κB activation, was induced by doxycycline in both HCT116p53<sup>−/−</sup> and HCT116p53<sup>+/−</sup> cells (Fig. 8B), and doxycycline-induced phosphorylation on Ser<sup>20</sup> was not detected in HCT116p53<sup>−/−</sup> cells expressing a transfected IxBoM (Fig. 8C), possibly because of the lack of detectable p53 protein. Together, these results appeared to suggest that doxycycline-induced phosphorylation of Ser<sup>20</sup> on p53 significantly increase the level of p53, thus extending its activity to initiate proapoptotic signaling cascades. However, it is unclear whether doxycycline-induced activation of NF-κB and Ser<sup>20</sup> phosphorylation on p53 is regulated independently or Ser<sup>20</sup> phosphorylation on p53 is regulated by a NF-κB-induced kinase.

Proteasome Inhibitor Abrogates Both NF-κB and p53 Activation—PS-341, a proteasome inhibitor, blocks NF-κB activation by preventing degradation of IκBα proteins (70, 71), and it may also increase the stability of p53 by inhibiting proteasome. Therefore, we used it at a nontoxic concentration to test whether the inhibition of NF-κB activity resulted in suppression of p53 expression and activity. MDAPanc-28/Puro cells were treated with 100 nM of PS-341 for specified periods. The results showed that DNA binding activity of both RelA/p50 heterodimer and p53 was reduced after 12 h of PS-341 treatment and that the inhibitory effect of PS-341 on the activation of these transcription factors was further enhanced in a time-dependent manner (Fig. 9A). The expression of p53 and p21<sup>vasf</sup> was down-regulated in a time-dependent manner after PS-341 treatment (Fig. 9, B and C), and these results are consistent with the inhibition of NF-κB- and p53-DNA binding activity. Fig. 9D shows a PS-341 dose-dependent inhibition of doxycycline-mediated apoptosis in MDAPanc-28/Puro cells. Thus, our results suggested that pharmacological proteasome inhibitors,
**Fig. 4.** p53 function is essential in doxycycline-mediated apoptosis. The p53 status of the HCT116 cells was confirmed by Northern (A) and Western blot (B) analyses. C, doxycycline-mediated cytotoxicity is p53-dependent. HCT116p53−/− and HCT116p53+/− cells were incubated with 50 μg/ml of doxycycline. At each indicated time interval, cells were collected, and the number of viable cells was determined as described under "Experimental Procedures." D, doxycycline induces NF-κB activation in HCT116p53−/− and HCT116p53+/− cells. NF-κB, p53, and Oct-1 EMSAs were performed using the nuclear extracts isolated from HCT116p53−/− and HCT116p53+/− cells stimulated with doxycycline at the various time intervals indicated. E, doxycycline-induced expression of PUMA is p53-dependent. RNA isolated from HCT116p53−/− and HCT116p53+/− cells stimulated with doxycycline for various intervals as indicated were analyzed by Northern blotting using p53 and PUMA probes. F, IκBαM completely suppresses doxycycline-induced NF-κB and p53 activation. NF-κB, p53, and Oct-1 EMSAs were performed using the nuclear extracts isolated from HCT116p53−/− cells transfected with a control vector (Ctl) and with IκBαM expression plasmid (IκBαM) and with doxycycline for the times indicated. G, IκBαM inhibits doxycycline-induced expression of p53 and PUMA. RNA was isolated from HCT116p53−/− cells (lanes 1–3) and HCT116p53+/− cells transfected with IκBαM expression plasmid (lanes 4–6), which were stimulated with 50 μg/ml of doxycycline for the indicated times and analyzed by Northern blotting with p53 and PUMA cDNA probes. The same blot was washed and rehybridized to a gapdh cDNA probe. H, IκBαM and lack of wild-type p53 inhibit doxycycline-induced apoptosis in HCT116p53−/− cells. HCT116p53+/− cells (lanes 1–5), HCT116p53−/− cells (lanes 6–10), and HCT116p53+/− cells transfected with IκBαM (lanes 12–16) were stimulated with 50 μg/ml of doxycycline for the indicated times, and DNA fragmentation assays were performed. Lanes 11 and 17 are DNA size markers.

**Fig. 5.** Doxycycline-mediated NF-κB and p53 activation requires IKK function. A, doxycycline-mediated NF-κB and p53 activation is inhibited in IκBα−/−, IκBβ−/−, and relA−/− murine fibroblasts were stimulated with or without 50 μg/ml of doxycycline for 24 h, and nuclear extracts were isolated and subjected to EMSA using NF-κB, p53, and Oct-1 probes. B, re-expression of RelA induces p53 DNA-binding activity, relA−/− murine fibroblasts were infected with RelA retrovirus and control retrovirus (puromycin). Forty-eight hours after infection, cell extracts were isolated and subjected to EMSA using NF-κB, p53, and Oct-1 probes, and immunoblotting with anti-p53, p21waf1, and β-actin antibodies.

**DISCUSSION**

Apoptosis is regulated by the integration of both proapoptotic and antiapoptotic signals. Why environmental stress or therapeutic agents induce apoptosis in some cancer cells but not in others is unclear. Therefore, delineating proapoptotic and antiapoptotic signaling cascades should provide insight for the control mechanisms of apoptosis. NF-κB and a number of its downstream target genes have been demonstrated to play a critical role in modulating resistance to apoptosis in response to many stimulations and cancer therapeutic agents (6, 72). Thus, inhibitors of NF-κB activation have a potential use in overcoming the resistance to apoptosis induced by various anticancer agents (73). However, accumulating evidence show apoptosis-promoting functions of NF-κB, although how NF-κB functions as a proapoptotic factor and which downstream target genes are induced by NF-κB activation to initiate proapo-
NF-κB regulated proapoptotic signaling cascades. In response to superoxide stimulation, NF-κB is induced which in turn activates p53-dependent apoptotic pathways.

We investigated the role of NF-κB and p53 in doxycycline-mediated apoptosis and have demonstrated the following: (i) doxycycline increases superoxide generation and subsequently activates NF-κB; (ii) doxycycline-induced p53 activation is inhibited by IκBα overexpression and the lack of RelA, IKK1, or IKK2; (iii) re-expression of RelA/p65 induces p53 activation in relA−/−fibroblasts and superoxide-inducible NF-κB activation is not inhibited in HCT116p53−/−cells; (iv) superoxide-induced p53 activation leads to the expression of its downstream target genes p21 and PUMA; (v) overexpression of IκBα or loss of wild-type p53 function postponed doxycycline-mediated apoptosis.

Fig. 6. Doxycycline-induced p53 expression is NF-κB-dependent. MDAPanc-28/Puro (lanes 1–3) and MDAPanc-28/IκBαM (lanes 4–6) cells were stimulated with doxycycline for the indicated times. A, RNA isolated from MDAPanc-28/Puro (Puro; lanes 1–3) and MDAPanc-28/IκBαM (IκBαM; lanes 4–6) cells stimulated with 50 μg/ml of doxycycline for the times indicated was analyzed by Northern blotting using p53 and gapdh probes. B, nuclear extracts were isolated from MDAPanc-28/Puro and MDAPanc-28/IκBαM cells, stimulated with 50 μg/ml of doxycycline for the indicated times, and subjected to Western blot analysis using an anti-p53 monoclonal antibody. The membrane was then reprobed for expression of β-actin for a loading control. C, doxycycline-mediated increase of p53 protein stability is NF-κB-dependent. Panc-28/Puro and Panc-28/IκBαM cells were unstimulated or were stimulated with 50 μg/ml of doxycycline for 24 h, followed by the pulse-and-chase experiments for indicated times. To equilibrate the detection of p53, 25-μg and 200-μg aliquots of the crude cell extracts from the cells with or without doxycycline stimulation, respectively, were used for p53 immunoprecipitation. D, graphic representation of p53 half-lives as determined by phosphorimaging. E, MDAPanc-28/Puro and Panc-28/IκBαM cells were treated with either PBS or 50 μg/ml of doxycycline for 24 h. After treatment, protein synthesis was blocked by cycloheximide, then nuclear proteins were isolated 2, 4, and 8 h after the addition of cycloheximide and analyzed by Western blotting with monoclonal antibodies to p53. To equilibrate the detection of p53, 25 μg of protein samples from MDAPanc-28/Puro cells and 100 μg from MDAPanc-28/IκBαM were used for Western blots with monoclonal antibodies for p53. F, graphic representation of p53 half-lives as determined by an image analyzer. Levels of p53 were calculated as a percentage of the 0-h time interval.
**Fig. 8. Phosphorylation of p53 protein is NF-κB-dependent.** A, nuclear extracts from MDAPanc-28/Puro cells stimulated with or without 50 μg/ml of doxycycline for the indicated times were subjected to immunoprecipitation with an anti-p53 monoclonal antibody to obtain similar level of p53 proteins and followed by Western blot analysis using an anti-Ser20 phosphorylated p53-specific monoclonal antibody. B and C, HCT116p53−/−, HCT116p53+/− cells, and HCT116p53+/− cells transfected either with a control plasmid or IκBaM expression plasmid, were stimulated with 50 μg/ml of doxycycline for the indicated times. The extracts from these cells as indicated were subjected to Western blot analysis using an anti-Ser20 phosphorylated p53-specific monoclonal antibody and an anti-IκBaM antibody. Bottom, the membrane was reprobed with an anti-β-actin antibody as a loading control.

**Fig. 9. Proteasome inhibitor PS-341 inhibits apoptosis.** A, PS-341 inhibits NF-κB and p53 activation and subsequently induction of apoptosis. Our results, summarized in Fig. 9E, reveal a possible mechanism by which NF-κB regulates proapoptotic signaling cascades by activating p53-dependent apoptotic pathways in doxycycline-induced cell death. Our study also suggests that NF-κB inhibitor, when used as adjuvant therapy, can block antioxidant agent-induced wild-type p53 activity and apoptosis, and that the therapeutic response may be effectively enhanced by using chemotherapeutic regimens and adjuvants to target the appropriate genetic defects in cancers.

Previous studies suggested that oxidative stress is involved in induction of NF-κB activation. However, several reports showed that H$_2$O$_2$-induced NF-κB activation is highly cell type-dependent, which may reflect difference in ROS metabolism (74). Tetracycline is known for preferential inhibition of mitochondrial protein synthesis and decreases the level of cytochrome c oxidase, the key components of electron transport chain (53, 54). It was thought that the reduction of the synthesis of cytochrome c oxidase may lead to a disruption of electron transport function and lead to electron leakage from the respiratory chain to O$_2$ thus resulting elevated levels of superoxide radicals. Our results supported this notion and showed that doxycycline induced superoxide formation, which may in turn induce NF-κB activation. Recently, Hayakawa et al. (75) showed that a commonly used antioxidant, NAC, inhibited TNF-induced NF-κB activation independently of antioxidative...
function by lowering the affinity of TNF receptor to its ligand. The recent finding raised question about the specificity of NAC as antioxidant and the role of ROS as a mediator for TNF-induced NF-κB activation. Therefore, whether or not NAC functioned as an antioxidant in blocking doxycycline-induced NF-κB activation is unclear. The role of doxycycline-induced superoxide in activation of NF-κB remains an ongoing study.

Transcription factor NF-κB can regulate both pro- and antioxidant signaling pathways; however, less is known about the mechanism by which NF-κB induces apoptosis. Kasibhatla et al. (24, 25) showed that activation of the two transcription factors NF-κB and AP-1 is crucially involved in FasL expression induced by etoposide, teniposide, and UV irradiation. There are a number of reports described that show the NF-κB-mediated proapoptosis involves up-regulation of p53 (76–78). For example, NF-κB may promote an apoptotic response in striatal medium-sized neurons to excitotoxic insult through up-regulation of c-Myc and p53 (76). However, it is unclear how p53 is up-regulated by NF-κB activation. Ryan et al. (79) reported that expression of p53 induced NF-κB activation in Saos-2 cell line transfected with a tetracycline-inducible p53 expression vector. In our analysis, doxycycline-induced NF-κB activation is not inhibited in HCT116p53−/− colon cancer cells. Activation of p53 completely inhibited in MDA Panc28 pancreatic cancer cells and HCT116 colon cancer cells expressing IkBaM and in IkKα deficient mouse fibroblasts. Re-expression of RelA in rela−/− mouse fibroblasts induces p53 activity and expression of its downstream target gene p21waf1, further indicating that NF-κB activation induces p53 in response to ROS. It is possible that this difference may be due to the different cell lines and the amount of inducing agents used in the experiments. Similarly, the difference in growth inhibition between MDA Panc-28/IxBαM and HCT116p53−/− cells may be cell-specific. It is also possible that p63 and p73, the members of the tumor suppressor p53 family, may partially compensate the p53 function in HCT116p53−/− and HCT116p53−/−; cells. Dr. Xiangwei Wu (Baylor College of Medicine, Houston, TX) for the kind gift of p65/NF-κB retroviral expression vector. We thank Dr. Mien-Chie Hung (The University of Texas MD Anderson Cancer Center) for critical reading of the manuscript. We also thank Ann Sutton for editorial assistance.

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