p21^{Cip1} Protection against Hyperoxia Requires Bcl-X_L and Is Uncoupled from Its Ability to Suppress Growth

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The cyclin-dependent kinase inhibitor p21^{Cip1/Waf1/Sdi1} protects the lung against hyperoxia, but the mechanism of protection remains unclear because loss of p21 does not lead to aberrant cell proliferation. Because some members of the *Bcl-2* gene family have been implicated in hyperoxia-induced cell death, the current study investigated their expression as well as p21-dependent growth suppression and cytoprotection. Conditional overexpression of full-length p21, its amino-terminal cyclin-binding (p21^{1-82NLS}) domain or its carboxy-terminal PCNA-binding (p21⁷⁶⁻¹⁶⁴) domain inhibited growth of human lung adenocarcinoma H1299 cells, but only the full-length protein was cytoprotective. Low levels of p21 inhibited cell proliferation, whereas higher levels were required for protection. Expression of the anti-apoptotic protein Bcl-X_L declined during hyperoxia but was maintained in cells expressing p21. RNA interference (RNAi) knockdown of Bcl-X_L enhanced hyperoxic death of cells expressing p21, whereas overexpression of Bcl-X_L increased cell survival. Consistent with growth suppression and cytoprotection requiring different levels of p21, hyperoxia inhibited PCNA expression in p21^{+/+} and p21^{+/-} mice but not in p21^{-/-} mice. In contrast, p21 was haplo-insufficient for maintaining expression of Bcl-X_L and protection against hyperoxia. Taken together, these data show that p21-mediated cytoprotection against hyperoxia involves regulation of Bcl-X_L and is uncoupled from its ability to inhibit proliferation. (*Am J Pathol* 2006, 168:1838-1847; DOI: 10.2353/ajpath.2006.051162)

Supplemental oxygen (hyperoxia) is an important therapeutic used to increase oxygen levels in peripheral tissues of patients suffering from respiratory distress.¹ Unfortunately, cytotoxic reactive oxygen species are produced during exposure that injures and kills alveolar microvascular endothelial and type I epithelial cells.^{2,3} Antioxidant molecules offer the first line of defense by their ability to detoxify reactive oxygen species.⁴ However, oxidative damage to DNA and other macromolecules occurs when these antioxidant defenses fail. Consistent with cellular responses to other types of DNA damage, hyperoxia stimulates expression of the tumor suppressor protein p53, which regulates expression of genes involved in cell cycle arrest, DNA repair, and apoptosis. Although hyperoxia also stimulates expression of the cyclin-dependent kinase inhibitor p21, the growth arrest and DNA damage (GADD) 45 gene, Bax, and other downstream p53 targets, many are also induced in p53-deficient mice.^{3,5-8} This lack of dependence on p53 may explain why p53-deficient mice are surprisingly not sensitive to hyperoxia.^{3,6} In contrast, p21-deficient newborn and adult mice exhibit marked increased sensitivity to hyperoxia, but the mechanisms by which p21 protects against hyperoxia remain undefined.^{5,9,10}

One mechanism by which p21 might protect against hyperoxia is by preventing replication of damaged DNA. The amino-terminal domain of p21 binds and controls activities of cyclin/cdk complexes involved in the G1-S transition, whereas the carboxy terminus binds proliferating cell nuclear antigen (PCNA) and inhibits PCNA-dependent DNA replication.^{11,12} Both amino- and carboxy-terminal proteins exert growth arrest when independently expressed in cell lines. In epithelial cell lines, hyperoxia

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stimulates p53-dependent expression of p21 that exerts G1 growth arrest by inhibiting cyclin E-dependent kinase activity.^{10,13,14} In the absence of p21, cells exit G1 and progressively arrest in S and G2/M phases of the cell cycle. G1 growth arrest could be restored in p53-deficient human lung adenocarcinoma H1299 cells by conditional overexpression of enhanced green fluorescent protein (EGFP) fused to the amino terminus of p21 (EGFP21).¹⁵ Conditional overexpression of the amino-terminal cyclin-binding domain p21¹⁻⁸² with addition of a nuclear localization signal (NLS) or the carboxy-terminal PCNA-binding domain p21⁷⁶⁻¹⁶⁴ containing the endogenous NLS also restored G1 arrest. Consistent with G1 arrest protecting against hyperoxia, HCT116/p21^{-/-} colon carcinoma cells exhibited increased sensitivity to hyperoxia compared with the parental wild-type line that expressed p21.¹⁰ Although p21-deficient newborn and adult mice are sensitive to hyperoxia,^{5,9} it is intriguing that the mitotic index of the adult lung is less than 2%.¹⁶ Moreover, loss of p21 increased the sensitivity of alveolar type I epithelial cells to hyperoxia. Because type I cells are the most terminally differentiated alveolar cell and are widely believed to be incapable of mitosis, p21-mediated growth suppression may not be required for cytoprotection.

Recent studies have led to an appreciation that members of the Bcl-2 gene family control cell survival and death during hyperoxia, even though hyperoxia induces mixed apoptotic and necrotic cell death.¹⁷⁻¹⁹ The distinction between apoptosis and necrosis may not be mutually exclusive, particularly during hyperoxia when continuous oxidative stress over days could promote necrosis by inhibiting apoptotic pathways from being completed. Initial studies in mice revealed that hyperoxia stimulates expression of Bcl-X_L but not Bcl-2, Bak, or Bad.^{3,8,20} Consistent with Bax and Bak being a critical mediator of hyperoxia-induced cell death, fibroblasts derived from Bax^{-/-}Bak^{-/-} mice exhibited increased resistance to hyperoxia.¹⁸ Hyperoxia also stimulated caspase-8-dependent cleavage of Bid to t-Bid.¹⁷ As predicted if Bax were activated, overexpression of the anti-apoptotic protein Bcl-X_L in Rat1a cells protected against hyperoxia-induced DNA fragmentation, lactate dehydrogenase (LDH) release, and cell death.¹⁸ However, this observation remains controversial because adenoviral-mediated delivery of Bcl-X_L to mice or A549 cells did not protect against hyperoxia.¹⁷ The anti-apoptotic protein Bfl-1/A1 also protects against hyperoxia, because its elevated expression in transgenic mice overexpressing IL-11 in airway epithelium is responsible for their increased resistance to hyperoxia.²¹ Thus, some members of the Bcl-2 family control hyperoxia-induced cell death.

An alternative method by which p21 might protect against hyperoxia is through its ability to block apoptosis. It can inhibit p53-dependent apoptosis in mouse embryo fibroblasts and melanoma cells transfected with adenovirus expressing p53.²² Consistent with that study, mouse embryo fibroblast cells lacking p21 express higher levels of Bax and are more susceptible to hydrogen peroxide killing.²³ Increased sensitivity of HCT116/p21^{-/-} cells to the chemotherapeutic drug daunomycin

has also been associated with enhanced expression of p53 and an imbalance in the Bax-to-Bcl-2 ratio toward apoptosis.²⁴ Importantly, this enhanced sensitivity was ameliorated by pifithrin- α , a small molecule inhibitor of p53 transcriptional activity. Likewise, genetic disruption of PUMA or Bax reduced sensitivity of HCT116/p21^{-/-} cells to adriamycin or hypoxia.²⁵ Because loss of p53 does affect sensitivity to hyperoxia, it is unclear whether p21 protects by modifying p53-dependent apoptosis.

In the current study, we use p53-deficient H1299 cells that conditionally express p21 and inbred p21-deficient mice to investigate how p21 protects against hyperoxia. Our studies identify a novel mechanism by which low levels of p21 inhibit proliferation, whereas higher levels protect against hyperoxia by maintaining expression of Bcl-X_L.

Materials and Methods

Mice and Exposure Conditions

Adult (8 to 12 weeks old) C57Bl/6J wild-type and p21-deficient mice were exposed to room air or hyperoxia (100% O₂) by placing the cages inside a Plexiglas chamber through which oxygen was delivered as previously described.²⁶ Animals were anesthetized by intraperitoneal injection with 0.13 mg/g sodium pentobarbital. Compliance measurements were performed on anesthetized mice as previously described.²⁷ The lungs were lavaged 10 times with saline, and total protein was quantified using the BCA method (BCA Protein Assay; Pierce, Rockford, IL), whereas total number of cells in the bronchoalveolar lavage fluid was quantified in the first lavage using a hemocytometer. LDH activity was measured by following NADH oxidation to NAD⁺ in the following reaction. Briefly, 0.8 ml of pyruvate solution (0.6 mmol/L pyruvic acid, 40.2 mmol/L K₂HPO₄, and 10.3 mmol/L KH₂PO₄, pH 7.5) was added to 20 ml of 0.24 mmol/L NADH solution. Pyruvate/NADH solution was then added to bronchoalveolar lavage fluid (50:50), and decreased absorbency was measured at 340 nm. Mice were then euthanized by intraperitoneal injection with 100 mg/kg sodium pentobarbital. The University of Rochester Committee on Animal Resources approved all exposures and handling of the mice.

RNase Protection Assay

Cell lysate was harvested in 4 mol/L guanidine isothiocyanate, 0.5% N-laurylsarcosine, 20 mmol/L sodium citrate, and 0.1 mol/L 2-mercaptoethanol. RNA was isolated using phase-lock gel columns (Eppendorf, Westbury, NY) and resuspended in diethylpyrocarbonate-treated water. RNase protection assays were performed with a customized mouse probe template kit according to the manufacturer's instructions (PharMingen, San Diego, CA) and as previously described.⁸ Protected products were separated on a 6% acrylamide-8 mol/L urea sequencing gel, dried, and visualized by exposure on PhosphorImager screens. Band intensities were quan-

tified with ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and normalized to L32 expression.

Immunohistochemistry

Lungs were inflation fixed through the trachea with 10% neutral buffered formalin. Lungs were dehydrated through a series of ethanol, cleared in xylene, and embedded in paraffin. Sections were deparaffinized and rehydrated through a series of ethanol before antigen retrieval by boiling in 10 mmol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was quenched with 3% H₂O₂, and sections were blocked and incubated overnight with rabbit anti-Bcl-X_L (1:250; Cell Signaling, Beverly, MA) or IgG control. Tissues were incubated with biotinylated goat anti-rabbit secondary antibody, and signal was amplified using the rabbit Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were reacted with 3,3'-diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin. Images were visualized with a Nikon E800 microscope (Melville, NY) and captured with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Quantification measurements were performed using Metamorph analysis software (Molecular Devices Corp., Sunnyvale, CA). Bcl-X_L staining was quantified and normalized to the number of blue nuclei per field.

Cell Culture

Human lung adenocarcinoma H1299 cells were cultured in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco, Grand Island, NY), and 20 µg/ml gentamicin (Cellgro, Herndon, VA). Cells were maintained in tissue culture flasks and exposed to normoxia (room air with 5% CO₂) or hyperoxia (95% O₂ with 5% CO₂) as previously described.²⁸ Cells with conditional transgene expression were incubated 2.0 µg/ml doxycycline (Sigma Chemical Company, St. Louis, MO) for 24 hours in normoxia before hyperoxia exposures. Viability was measured by trypan blue exclusion. Briefly, cells were plated in 60-mm dishes and exposed to room air or hyperoxia. Cells were trypsinized after exposure and resuspended in trypan blue:phosphate-buffered saline (50:50), and cells were counted on a hemocytometer.

Cell Lines

The EGFP21, p21^{1-82NLS}, p21⁷⁶⁻¹⁶⁴, and EGFP doxycycline-inducible constructs and stable H1299 cell lines have been previously described.²⁹ The p21¹⁻¹⁴⁰ and p21¹⁻⁸² domain sequences were generated by reverse transcriptase-polymerase chain reaction of RNA isolated from human colon carcinoma HCT116 cells using the same forward primer (5'-ATGTCAGAACCGCTGGG-3') and reverse primer (5'-TCACCCTGAAGAGTCTCCAGTCC-3') for p21¹⁻¹⁴⁰ and reverse primer (5'-TCAGGGCCCCGTGGGAAGGTAGAG-3') for p21¹⁻⁸². *EcoRI* sites were added to the 5' primer, and *BamHI* sites were

added to the 3' primers. The *EcoRI*- and *BamHI*-digested fragments were ligated into a pBIG2i vector that contained an amino-terminal Met-Flag coding sequence upstream of the *EcoRI* site and the internal ribosome entry site (IRES)-EGFP sequence from pIRES2-EGFP (Clontech, Palo Alto, CA) downstream of the *BamHI* site to produce Flag-tagged p21¹⁻¹⁴⁰ IRES-EGFP and Flag-tagged p21^{1-82NLS} IRES-EGFP. The human Bcl-X_L open-reading frame was amplified by reverse transcriptase-polymerase chain reaction using RNA obtained from H1299 cells with forward (5'-ATCTCTCAGAGCAACCGGA-3') and reverse primers (5'-TCATTTCGACTGAAGAGTGAGCC-3'). An *EcoRI* restriction site was added to the forward primer, and a *BglII* restriction site was added after the termination codon of the reverse primer. The amplified product was digested and also ligated into the pBig2i amino-terminal Met-Flag containing the downstream IRES-EGFP sequence to produce Flag-tagged Bcl-X_L IRES-EGFP. The plasmids were purified by Qiagen preparation (Qiagen Sciences, Valencia, CA) and transfected into H1299 cells using Genfect (Molecular, Columbia, MD). Cells were grown in 200 µg/ml hygromycin (Invitrogen, Carlsbad, CA), and stable clones were initially selected based on EGFP fluorescence after treatment with 2 µg/ml doxycycline (Sigma Chemical Company) and screened using a Nikon TE2000-E inverted epi-fluorescent microscope.

RNAi Transfections

Cells were plated in 60-mm dishes overnight and transfected with 100 nmol/L annealed Bcl-X_L or BLOCK-iT fluorescein isothiocyanate-conjugated oligonucleotides (Invitrogen) diluted in Opti-MEM I (Gibco) using Lipofectamine 2000. The Bcl-X_L target sequence (5'-AAGAGAATCACTAACCAGAGA-3') was homologous to nucleotides located -66 to -86 of the translation initiation site. After 24 hours, cells were washed and cultured in normal medium before exposures to hyperoxia.

Western Blot Analysis

Cells and whole lungs were lysed in 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 2 mmol/L ethylenediamine tetraacetic acid, 25 mmol/L sodium fluoride, 25 mmol/L sodium β-glycerophosphate, 0.1 mmol/L sodium vanadate, 0.1 mmol/L phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 0.3% IGEPAL CA-630, 0.1 µg/ml pepstatin A, 1.9 µg/ml aprotinin, and 2 µg/ml leupeptin. Protein concentrations were determined by the BCA method (Pierce). Cell lysates were diluted in 3× Laemmli buffer and boiled for 5 minutes. Laemmli at 1× contains 50 mmol/L Tris (pH 6.8), 1% β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, and 10% glycerol. The extracted protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Pall Life Sciences, East Hills, NY). The membranes were then incubated with anti-EGFP (1:1000; Clontech, Palo Alto, CA), anti-p21 clone CP36 (1:20), anti-p21 clone SX118

Figure 1. p21 inhibits proliferation during hyperoxia. **A:** Representative RNase protection analysis of RNA isolated from lungs of p21^{+/+}, p21^{+/-}, and p21^{-/-} mice exposed to hyperoxia for 72 hours. Each lane contains RNA from separate mice. **B:** Band intensities for p21 were quantified by phosphorimager analysis and graphed after normalizing to L32. Values represent means $\pm$ SD, $n = 4$ (* $P < 0.0001$). **C:** Western blot analysis of PCNA expression in p21^{+/+} mice exposed to room air (time 0) or 24, 48, and 72 hours of hyperoxia. Actin is used to control for loading of proteins. **D:** Western blot analysis of p21^{+/+}, p21^{+/-}, and p21^{-/-} mice exposed to hyperoxia for 72 hours. Blots are representative of three to four experiments with similar results.

(1:500; Pharmingen), anti-Flag (1:250; Sigma Chemical Company), anti-PCNA (1:1000; Zymed, San Francisco, CA), anti-Bcl-X_L clone 2H12 (1:500; Sigma Chemical Company), and anti-Bax clone N-20 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), with β -actin (1:1000, Sigma Chemical Company) as a loading control. Membranes were then incubated in horseradish peroxidase-conjugated secondary anti-mouse (1:4000; Southern Biotechnology, Birmingham, AL) or anti-rabbit (1:5000; Jackson ImmunoResearch, West Grove, PA) antibodies and visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

Values are means $\pm$ SD. Group means were compared by analysis of variance using Fisher's procedure post hoc analysis with StatView (Abacus Concepts, Berkeley, CA) software. $P < 0.05$ was considered significant.

Results

p21 Is Haplo-Insufficient for Protection against Hyperoxia but Not for Growth Suppression

To determine whether p21 expression during hyperoxia is controlled by gene dosage, p21^{+/+}, p21^{+/-}, and p21^{-/-} mice were exposed to hyperoxia for 72 hours. Total RNA was isolated from lungs, and p21 expression was analyzed by RNase protection assay (Figure 1A). As expected, p21 mRNA was readily detected in p21^{+/+} and p21^{+/-} lungs but not in p21^{-/-} lungs. Quantitative analysis revealed that p21^{+/-} mice expressed p21 at levels 59% of that detected in p21^{+/+} mice (Figure 1B). Studies in cardiomyocytes show that p21^{-/-} cells have increased PCNA levels and that this has direct effects on cell proliferation.³⁰ As assessed by mRNA expression of the replication-dependent histone H3.2, hyperoxia inhibits cell proliferation in adult p21^{+/+} mice but not in p21^{-/-}

Figure 2. p21 is haplo-insufficient for protection against hyperoxia. p21^{+/+}, p21^{+/-}, and p21^{-/-} mice were exposed to hyperoxia for 72 hours. **A:** p21^{+/+} and p21^{-/-} mice exhibited less survival compared with p21^{+/+} mice. **B:** Lung compliance declined in mice exposed to hyperoxia (* $P < 0.001$) and was significantly worse in p21^{+/-} and p21^{-/-} mice ($^{\dagger}P < 0.05$). **C:** Bronchoalveolar lavage protein increased in mice exposed to hyperoxia (* $P < 0.001$) and was significantly higher in p21^{+/-} and p21^{-/-} mice ($^{\dagger}P < 0.01$). **D:** LDH activity increased in mice exposed to hyperoxia (* $P < 0.02$) and was significantly higher in p21^{+/-} and p21^{-/-} mice ($^{\dagger}P < 0.02$). Values in **B**, **C**, and **D** represent means $\pm$ SD, $n = 5$.

mice.⁹ Consistent with those findings, PCNA levels declined in p21^{+/+} mice exposed to hyperoxia (Figure 1C). To determine whether gene dosage plays a role in growth suppression, the expression of PCNA was investigated in p21^{+/+}, p21^{+/-}, and p21^{-/-} mice exposed to hyperoxia for 72 hours. Although PCNA expression was comparable in p21^{+/+} and p21^{+/-} mice, it was markedly higher in p21^{-/-} mice (Figure 1D). These findings confirm previous work showing that p21 mice are haplo-sufficient for growth suppression.³¹

Because loss of p21 sensitizes mice to hyperoxia, several parameters of cell and tissue injury were assessed in p21^{+/+}, p21^{+/-}, and p21^{-/-} mice exposed to room air and hyperoxia. Cumulative survival analysis indicated that 92% of p21^{+/+} mice survived to 72 hours of hyperoxia, whereas only 54% of p21^{+/-} and 69% of p21^{-/-} mice were alive at that time (Figure 2A). Lung compliance was used to assess pulmonary function in mice exposed to room air and hyperoxia. Lung compliance was not significantly altered among mice with different p21 genotypes exposed to room air (Figure 2B). In contrast, lung compliance declined in p21^{+/+} mice exposed to hyperoxia and was significantly worse in p21^{+/-} and p21^{-/-} mice. Increases in protein or LDH activity in bronchoalveolar lavage fluid are indicators of edema and cell death, respectively. Although alveolar protein was not different in mice exposed to room air, it increased in p21^{+/+} mice and was significantly increased in p21^{+/-} and p21^{-/-} mice (Figure 2C). Likewise, LDH activity was similar in mice exposed to room air (Figure 2D). Although LDH activity remained low in p21^{+/+} mice exposed to hyperoxia, it markedly increased in p21^{+/-} and p21^{-/-} mice consistent with their enhanced sensitivity. These findings reveal that p21 is haplo-insufficient for protection against hyperoxia.

Conditional Expression of p21 Affects Growth and Survival of H1299 Cells

Because endogenous levels of p21 are not detected in p53-deficient H1299 cells exposed to hyperoxia, stable cell lines were created that conditionally express EGFP fused amino-terminal to p21 (EGFp21), the amino-terminal cyclin-dependent kinase binding domain (amino acids 1 to 82), the carboxy-terminal PCNA binding domain (amino acids 76 to 164), or EGFP by itself. Using these cell lines, we established that conditional overexpression of EGFp21, p21^{1-82NLS}, or p21⁷⁶⁻¹⁶⁴ restored G1 growth arrest during hyperoxia.¹⁵ To further assess the effect of these proteins on proliferation, cells were cultured for 4 days in the absence or presence of doxycycline (2 μ g/ml). Western blot analysis confirmed that doxycycline stimulated expression of EGFp21, p21^{1-82NLS}, p21⁷⁶⁻¹⁶⁴, or EGFP (Figure 3A). Cells were then cultured in the absence or presence of doxycycline for 4 days, harvested, and counted. As expected, overexpression of EGFp21 or its individual amino- and carboxy-terminal domains markedly inhibited cell growth, whereas overexpression of EGFP had little effect (Figure 3B). As measured by trypan blue dye exclusion or sub-G1 DNA content, reduced growth in the presence of doxycycline was not due to increased cell death (data not shown).

To assess whether conditional overexpression of these proteins protected against hyperoxia-induced cell death, cells were cultured in the absence or presence of doxycycline for 24 hours and then exposed to hyperoxia for 6 days. In the absence of doxycycline, cell viability was less than 10% (Figure 3C). Conditional overexpression of EGFp21 markedly protected against hyperoxia, whereas overexpression of p21^{1-82NLS}, p21⁷⁶⁻¹⁶⁴, or EGFP had little effect on viability. Cells lacking EGFp21 expression appeared small and spherical with poor adhesion, whereas cells expressing EGFp21 had multiple cellular extensions and were tightly adhered to the plates (Figure 3, D and E). Because the nuclear localization signal is located in the carboxy terminus, a viral NLS had been added to the p21¹⁻⁸² to enhance nuclear targeting. To exclude that nuclear targeting had not disrupted the cytoprotective effects of that domain, a stable line of H1299 cells that conditionally expressed p21¹⁻⁸² without an NLS (p21^{1-82ΔNLS}) was created. As previously reported,¹² removal of the NLS significantly attenuated the growth inhibitory activities of this domain. But it did not protect against hyperoxia (data not shown). This suggested that cytoprotection was mediated by the carboxy-terminal PCNA binding domain. However, conditional overexpression of a mutant p21 lacking only the PCNA-binding domain (p21¹⁻¹⁴⁰) also did not protect against hyperoxia (data not shown). Thus, only the full-length form of p21 is sufficient to protect against hyperoxia.

Cell Growth and Survival Require Different Levels of p21 Expression

The observation that overexpression of p21^{1-82NLS} or p21⁷⁶⁻¹⁶⁴ restored G1 arrest but did not protect against

Figure 3. Full-length p21 is required for protection against hyperoxia. H1299 cells with doxycycline-inducible expression of EGFp21, p21^{1-82NLS}, p21⁷⁶⁻¹⁶⁴, or EGFP were cultured in the absence (–) or presence (+) of 2 μ g/ml doxycycline (dox). **A:** Western blot analysis of cell lysates collected after 24 hours. **B:** Cells were cultured in the absence or presence of doxycycline (2 μ g/ml) for 4 days and counted. Graphs represent mean $\pm$ SD, $n = 3$ (* $P < 0.001$) relative to number of cells obtained in the absence of doxycycline. **C:** Cell viability of cultures exposed to hyperoxia for 6 days in the absence or presence of doxycycline. Values represent mean $\pm$ SD, $n = 4$ (* $P < 0.0005$) relative to cells cultured without doxycycline. **D** and **E:** Representative images of H1299+EGFp21 cells exposed to hyperoxia for 6 days in the absence or presence of doxycycline.

hyperoxia suggests that these processes may be uncoupled. To investigate further the relationship between growth suppression and cytoprotection, H1299 cells with conditional expression of EGFp21 were exposed to increasing amounts of doxycycline. As assessed by Western analysis, doxycycline stimulated dose-dependent expression of EGFp21 with maximal expression seen with 0.75 μ g/ml of the drug (Figure 4A). The cells were then treated with these doses of doxycycline and placed in room air for 4 days for proliferation studies or in hyperoxia for 4 days for survival studies. Although a dose of 0.25 μ g/ml doxycycline was sufficient to inhibit growth by 50% (normalized to un-

Figure 4. Growth arrest and survival require different levels of p21. H1299-EGFP21 cells were treated with increasing amounts of doxycycline (dox) for 24 hours. **A:** Cell lysates were collected and immunoblotted for EGFP21 and actin. **B:** Cell viability was measured after 4 days of hyperoxia, and values were graphed relative to cells cultured in the absence of doxycycline. Values represent mean $\pm$ SD, $n = 4$.

treated cells), 0.75 μg/ml was required to enhance survival by 50% (Figure 4B). These data indicate that low levels of p21 are required for growth suppression, whereas higher levels are needed for cytoprotection.

p21 Maintains the Expression of Bcl-X_L during Hyperoxia

Recent studies have led to an appreciation that members of the Bcl-2 gene family control cell survival and death during hyperoxia.¹⁸ To assess whether p21 controls expression of these proteins, H1299-EGFP21 cells were cultured in the absence or presence of doxycycline for 24 hours and then exposed to hyperoxia for 2, 4, and 6 days. Although overexpression of EGFP21 for 24 hours (time 0) did not affect expression of Bcl-X_L, it attenuated the loss of Bcl-X_L in cells exposed to hyperoxia (Figure 5). In contrast, Bax expression modestly increased during hyperoxia but was not affected by expression of EGFP21. Because individual domains of p21 were insufficient to protect against hyperoxia, their ability to maintain expression of Bcl-X_L during hyperoxia was evaluated. Although conditional overexpression of EGFP21 maintained expression of Bcl-X_L in cells exposed to hyperoxia (Figure 6A), conditional overexpression of p21^{1-82NLS}, p21⁷⁶⁻¹⁶⁴, or EGFP was insufficient to prevent the loss of Bcl-X_L (Figure 6, B–D). Thus, only full-length forms of p21 are sufficient to prevent the loss of Bcl-X_L during hyperoxia.

Figure 5. EGFP21 maintains Bcl-X_L expression during hyperoxia. H1299-EGFP21 cells were cultured in the presence or absence of 2.0 μg/ml doxycycline for 24 hours and placed into hyperoxia for 0, 2, 4, or 6 days. Cell lysates were collected and immunoblotted for p21, Bcl-X_L, Bax, and actin. Blots are representative of three experiments with similar results.

p21-Dependent Expression of Bcl-X_L Is Sufficient to Protect against Hyperoxia

Because conditional expression of EGFP21 blocked loss of Bcl-X_L during hyperoxia, RNAi methods were used to reduce levels of Bcl-X_L in these cells. H1299-EGFP21 cells were cultured in the absence or presence of doxycycline for 24 hours, transfected with annealed siRNA oligonucleotides, and then exposed to hyperoxia for 4 days. Western blot analysis revealed that Bcl-X_L expression was significantly reduced in cells transfected with

Figure 6. Full-length p21 is required for maintaining Bcl-X_L during hyperoxia. H1299 cells with inducible EGFP21 (**A**), p21^{1-82NLS} (**B**), p21⁷⁶⁻¹⁶⁴ (**C**), or EGFP (**D**) expression were cultured in the presence or absence of 2.0 μg/ml doxycycline for 24 hours and kept in room air or placed into hyperoxia for 4 days. Cell lysates were collected and immunoblotted for p21, EGFP, Bcl-X_L, and actin. Blots are representative of three experiments with similar results.

Figure 7. Bcl-X_L protects against hyperoxia. **A:** Western blot analysis of H1299-EGFP21 cells mock transfected (M) or transfected with 100 nmol/L fluorescent nontargeting (F), or Bcl-X_L-targeting (B) oligonucleotides. **B:** Bcl-X_L-targeting oligonucleotides significantly reduced survival of H1299-EGFP21 cells exposed to 4 days of hyperoxia in the absence (**P* < 0.008) or presence (**P* < 0.005) of doxycycline. **C:** Western blot analysis of H1299-Bcl-X_L cells cultured in the absence or presence of doxycycline (2 μg/ml) for 24 hours. **D:** Addition of doxycycline to H1299-Bcl-X_L cells enhanced cell survival after 4 days (**P* < 0.06) and 6 days (**P* < 0.01) of hyperoxia.

siRNAs against Bcl-X_L but not in mock-transfected cells or in cells transfected with fluorescently labeled oligonucleotides (Figure 7A). Based on visual inspection of cells transfected with fluorescently labeled oligonucleotides, transfection efficiency exceeded 95% (data not shown). Cell viability was then assessed by trypan blue dye exclusion. Although siRNAs against Bcl-X_L reduced viability of cells cultured in the absence of doxycycline, they markedly reduced survival of H1299-EGFP21 cells treated with doxycycline (Figure 7B).

To confirm that Bcl-X_L was a relevant target of p21-mediated cytoprotection against hyperoxia, Flag-tagged Bcl-X_L followed by an IRES and EGFP was conditionally overexpressed in the parental H1299 cell line. Several stable clones were obtained, and expression of Flag-tagged Bcl-X_L and EGFP was analyzed by Western blot analysis (Figure 7C). As shown for one clone, the addition of doxycycline markedly stimulated expression of Flag-tagged Bcl-X_L and EGFP by 24 hours. To assess whether conditional overexpression of Flag-tagged Bcl-X_L protected against hyperoxia-induced cell death, cells were cultured in the absence or presence of doxycycline for 24 hours and then exposed to hyperoxia for 2, 4, and 6 days. In the absence of doxycycline, cell viability progressively declined during hyperoxia. In contrast, doxycycline-dependent stimulation of Bcl-X_L markedly protected H1299 cells against hyperoxia. These data reveal that p21 protects against hyperoxia by maintaining expression of Bcl-X_L.

p21 Is Haplo-Insufficient for Maintaining Expression of Bcl-X_L during Hyperoxia

Western blot analyses using whole-lung extracts were performed to determine whether p21 maintained expression of Bcl-X_L *in vivo*. Bcl-X_L was readily detected in p21^{+/+} and p21^{-/-} mice exposed to room air (Figure 8A). Although Bcl-X_L levels remained constant in p21^{+/+}

Figure 8. p21 maintains expression of Bcl-X_L during *in vivo* exposure to hyperoxia. p21^{+/+} and p21^{-/-} mice were exposed to hyperoxia for 24, 48, and 72 hours. **A:** Lung homogenates were immunoblotted for Bcl-X_L and actin. Blots are representative of three separate exposures. **B:** Expression of Bcl-X_L in p21^{+/+}, p21^{+/-}, and p21^{-/-} mice exposed to hyperoxia for 72 hours was quantified, normalized to actin, and graphed. Values represent mean ± SD, *n* = 3 (**P* < 0.005). p21^{+/+} (**C** and **E**) and p21^{-/-} (**D** and **F**) lungs exposed to 72 hours of hyperoxia were immunostained for Bcl-X_L. **Filled arrows** denote type II cells, and **open arrows** denote microvascular endothelial and type I epithelial cells.

mice exposed to hyperoxia, they modestly declined by 72 hours in p21^{-/-} mice. To assess whether loss of Bcl-X_L was controlled by gene dosage, its expression was closely evaluated in p21^{+/+}, p21^{+/-}, and p21^{-/-} mice exposed to hyperoxia for 72 hours. As shown by the representative Western analysis and by graphic analysis after normalization to expression of actin, Bcl-X_L expression was significantly lower in p21^{+/-} and p21^{-/-} mice compared with p21^{+/+} (Figure 8B). Cellular expression was localized by immunostaining lungs of p21^{+/+} and p21^{-/-} mice exposed to hyperoxia for 72 hours. Strong staining was detected throughout the parenchyma of p21^{+/+} lungs, with minimal expression detected in fibroblasts underlying larger conducting airways and endothelial cells of large arteries and veins (Figure 8, C and E). Relative to p21^{+/+} mice, quantification using analysis software revealed a 32% decrease in Bcl-X_L throughout the lungs of p21^{-/-} mice (*P* < 0.01) (Figure 8D). Increased magnification revealed numerous Bcl-X_L-positive cells in the parenchyma of p21^{+/+} lungs, whereas p21^{-/-} lungs had decreased or absent staining (Figure 8, D and E). Consistent with mortality being attributed to death of microvascular endothelial and type I epithelial cells, reduced staining was most notable in these cell populations. In contrast, staining intensity was stronger in alveolar type II cells, which are more tolerant of hyperoxia. These data

reveal that p21 is haplo-insufficient for maintaining expression of Bcl-X_L during *in vivo* exposure to hyperoxia.

Discussion

The cyclin-dependent kinase inhibitor p21 is the predominant mediator of the G1 checkpoint when cells are damaged. Consistent with checkpoints protecting cells against genotoxic stress, loss of p21 leads to enhanced cell death. Although growth arrest is cytoprotective, it is unclear how checkpoint proteins integrate cell cycle arrest with DNA repair and cell survival. Using hyperoxia as a persistent model of oxidative stress, we provide evidence that p21-mediated cell cycle arrest and cytoprotection are uncoupled and require different levels of p21. Moreover, p21 protects against hyperoxia by maintaining expression of Bcl-X_L that otherwise would decline over time. Thus, low levels of p21 inhibit growth, whereas higher levels promote an anti-apoptotic phenotype thereby allowing repair of damaged DNA that might otherwise be a stimulus for apoptosis. These findings help explain how p21 can protect the mitotically inactive adult lung against hyperoxia.

An important observation in the current study is that overexpression of either amino- or carboxy-terminal domains inhibited growth of H1299 cells but were insufficient to protect against hyperoxia. It is well established that ionizing radiation, anticancer drugs, hyperoxia, and other forms of genotoxic stress stimulate p53-dependent expression of p21, which executes the G1 checkpoint via inhibition of G1 cyclin/cdk complexes and PCNA-dependent replication. Damaged cells that fail to express p21 continue to replicate DNA and eventually die.³² However, cells that fail to express p21 during hyperoxia progressively accumulate in S and G2/M phase through a presently undefined mechanism. One hypothesis is that the persistent production of reactive oxygen species during hyperoxia damages DNA or proteins so severely that DNA replication cannot continue. Alternatively, hyperoxia activates an intra-S phase checkpoint pathway.³³ Either way, the genomic DNA of cells in S phase is likely to be highly sensitive to oxidative attack, because histones that can protect against damage are removed during replication. Although it is unclear whether growth arrest in S phase leads to more damage, the present findings reveal that p21-mediated protection is not attributed to its ability to simply prevent cells from accumulating in S phase.

One of the most documented methods by which p21 protects cells is through its ability to prevent p53-dependent apoptosis. This concept was first reported with fusions of different colorectal carcinoma cells that exhibited growth arrest or apoptosis when forced to express p53.³⁴ Cells that growth arrested in the presence of p53 now underwent apoptosis when p21 was genetically ablated. Consistent with p21 countering p53-dependent apoptosis, increased apoptosis is seen in p21-deficient mouse embryo fibroblasts forced to express p53.²² Moreover, increased sensitivity of HCT116/p21^{-/-} cells to daunomycin, adriamycin, or hypoxia was prevented by treating cells with pifithrin- α , a small molecule inhibitor of p53, or

by ablation of the p53-dependent apoptotic genes PUMA or Bax.^{24,25} Although HCT116/p21^{-/-} cells have higher levels of p53, their increased sensitivity to hyperoxia cannot be rescued by pifithrin- α or ablation of Bax or PUMA (R. Staversky, unpublished observations). The current observation that p21 protects p53-deficient H1299 cells against hyperoxia by maintaining expression of Bcl-X_L further supports the hypothesis that p21-mediated protection against hyperoxia is novel and independent of p53. At present, it is unclear why loss of p53 does not affect sensitivity to hyperoxia. A hypothesis that we favor is that hyperoxia is a chronic model of oxidative stress and damage that occurs over several days, during which time the pro-apoptotic functions of p53 could become compromised. Oxidative stress can disrupt p53 transcriptional activity.³⁵

Our observation that overexpression of Bcl-X_L protected p21-deficient cells helps clarify previous controversy as to why Bcl-X_L failed to protect A549 epithelial cells or adult mice against hyperoxia while strongly protecting Rat1a fibroblasts.^{17,18} In adult mice and A549 cells, hyperoxia stimulates expression of p21 that protects cells by maintaining expression of Bcl-X_L.^{9,14} Thus, addition of Bcl-X_L via adenovirus has no effect. In contrast, the p21 promoter is hypermethylated and transcriptionally inactive in Rat1a cells.³⁶ p21 is therefore unavailable to maintain endogenous levels of Bcl-X_L in Rat1a cells during hyperoxia. Thus, overexpression of Bcl-X_L in these cells does protect against hyperoxia. Obviously, formal proof that p21 protects lungs against hyperoxia through expression of Bcl-X_L requires the use of Bcl-X_L-deficient mice. Because Bcl-X_L deficiency is embryonic lethal, floxed Bcl-X_L mice have been created, which we will be using to investigate how Bcl-X_L protects individual cell populations of the lung against hyperoxia and other inhaled pollutants.³⁷

Another major finding in this study is that p21 blocks the inhibitory actions of hyperoxia on Bcl-X_L expression. The *Bcl-X* gene encodes at least five different Bcl-X isoforms generated by alternative splicing of mRNAs transcribed from multiple promoters.^{38–40} All isoforms share a large common amino terminus with functional specificity being dictated by small differences in the carboxy terminus. The large and most abundant isoform Bcl-X_L protects cells against apoptosis, whereas the short isoform Bcl-X_S antagonizes the anti-apoptotic effects of Bcl-X_L and Bcl-2. Bcl-X_{ΔTM} is an alternatively spliced anti-apoptotic isoform of Bcl-X_L that is expressed diffusely throughout the cytoplasm. A fourth isoform, Bcl-X_β, promotes apoptosis and is expressed in the cerebellum, thymus, and heart. A fifth isoform, Bcl-X_γ, blocks apoptosis of T cells. In addition to alternative splicing to create novel isoforms, the *Bcl-X* gene contains five distinct promoters located within 3.5 kb of the open-reading frame that control basal, tissue-specific, and steroid-responsive expression of these isoforms.^{41,42} Expression of the different isoforms is controlled by several stimuli acting through transcription factors, including Ets-1 and -2, GATA-1, NF- κ B, AP-1, and STAT-3 (for review, see Ref. 43). Hyperoxia can affect NF- κ B, AP-1, and STAT-3 activity. Because p21 can mediate estrogen-dependent

transcriptional regulation of the progesterone receptor, it might block transcriptional repression of the Bcl-X gene during hyperoxia.⁴⁴ Alternatively, p21 might bind and block degradation of Bcl-X_L. Precedence for this concept also exists in that p21 binds and markedly affects stability of PCNA.³⁰ Thus, p21 might affect expression of multiple Bcl-X isoforms at the level of transcription or protein stability.

In summary, this study showed that p21-mediated cell cycle arrest and cytoprotection are uncoupled and require different levels of p21. Our findings suggest a model in which low levels of damage stimulate p53-dependent expression of p21 that promotes G1 growth arrest. Increased damage or prolonged genotoxic stress, such as during hyperoxia, results in elevated levels of p21 that protect by maintaining expression of Bcl-X_L. Because hyperoxia is a model of persistent oxidative stress, our findings may also be relevant for ischemia/reperfusion injuries, diseases of chronic inflammation (Crohn's disease, bronchiectasis, rheumatoid arthritis, etc.), neuronal degeneration, and even the aging process.

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