Downregulation of PCNA potentiates p21-mediated growth inhibition in response to hyperoxia

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Gehen SC, Vitiello PF, Bambara RA, Keng PC, O’Reilly MA. Downregulation of PCNA potentiates p21-mediated growth inhibition in response to hyperoxia. Am J Physiol Lung Cell Mol Physiol 292: L716–L724, 2007. First published November 3, 2006; doi:10.1152/ajplung.00135.2006.—Prolonged exposure to hyperoxia inhibits cell proliferation in G1 via increased expression of p21. While p21 inhibits proliferating cell nuclear antigen (PCNA)-dependent DNA synthesis, it can also directly lower PCNA abundance; however, it is unclear whether loss of PCNA contributes to growth arrest. Here, we investigate how PCNA loss affects ability of p21 to exert G1 growth arrest of lung epithelial cells exposed to hyperoxia. In A549 cells that express p21 and growth arrest in G1 during hyperoxia, small interfering RNA (siRNA) knockdown of p21 led to G1 checkpoint bypass, increased cell death, and restoration of PCNA expression. Conditional overexpression of the PCNA binding domain of p21 in H1299 cells that do not normally express p21, or exposure to hyperoxia, caused a time-dependent loss of PCNA. Titrating PCNA levels using siRNA to approximate the low amount observed in cells expressing p21 resulted in S phase arrest. While lowering PCNA by itself caused S phase arrest, the combination of hyperoxia and siRNA against PCNA dramatically reduced PCNA abundance resulting in G1 arrest. G1 growth arrest was markedly enhanced upon the addition of p21 to these cells. Our findings suggest a model in which reducing expression of the abundant protein PCNA allows the less abundant protein p21 to be more effective at suppressing the processivity functions of remaining PCNA, thereby fully exerting the G1 checkpoint. Given that high p21 expression is often associated with lower PCNA abundance, our findings are suggestive of a global growth inhibitory mechanism involving p21-mediated PCNA suppression.

DNA damage; oxidative stress; cell cycle checkpoint; proliferating cell nuclear antigen

WHILE CLINICALLY NECESSARY to prevent tissue hypoxia, prolonged exposure to elevated levels of oxygen (hyperoxia) results in the enzymatic production of reactive oxygen species (ROS). Failure to detoxify ROS leads to a chronic state of oxidative stress and causes damage to all macromolecules resulting in cell injury and death (21). Oxidative DNA damage represents an especially critical challenge to the continued survival of cells exposed to hyperoxia. As with other forms of genotoxic stress, DNA damage caused by hyperoxia results in p53 stabilization and transcriptional activation of the cyclin-dependent kinase inhibitor p21 (16, 34).

In multiple cell line models, continuous exposure to hyperoxia results in p21 induction and G1 growth arrest that is maintained over days of exposure (18, 29, 35). Interestingly, p21 induction in BEAS-2B cells exposed to hyperoxia was associated with a relative increase in S phase-accumulated cells (3). Although consistent with other reports demonstrating S phase arrest in hyperoxia, it was not clear whether p21 was functional or activated a presently undefined intra-S phase checkpoint. On the other hand, a direct role for p21 in eliciting G1 arrest in hyperoxia has been demonstrated using genetic models including isogenic wild-type or p21−/− HCT116 cells and H1299 cells with conditional overexpression of p21 (16, 18). Thus, in multiple cell line models, activation and maintenance of the G1 checkpoint in hyperoxia is primarily mediated by the activity of p21.

p21 is a bifunctional protein with separate activities residing in the amino- and carboxy-terminal domains. Through aminoterminal interactions, p21 inhibits the kinase activity of cyclin/cdk complexes required for cell cycle progression (15). Through its unique carboxy-terminal domain, p21 binds to proliferating cell nuclear antigen (PCNA) and inhibits its function as a DNA polymerase processivity factor (7, 12). Since p21 has two distinct domains, G1 arrest could occur through amino-terminal and/or carboxy-terminal activities, although the relative importance of each domain in eliciting growth arrest may be cell type or damage dependent (5, 23, 36).

The role of p21 in activating and maintaining the G1 checkpoint in response to hyperoxia also requires clarification. Early studies revealed that inhibition of cyclin E/cdk2 activity in neonatal rat type II alveolar epithelial cells was likely due to the action of p21 (9). Although cell cycle progression was not directly assayed, this result is consistent with a role for the amino-terminal cdk-binding domain of p21 in hyperoxia-induced growth arrest. Consistent with p21 being responsible for the G1 checkpoint during hyperoxia, conditional overexpression of p21 or its amino-terminal and carboxy-terminal domains was sufficient to restore G1 arrest in p53-deficient H1299 cells exposed to hyperoxia (18). While expression of both domains caused cells to accumulate in G1, expression of the PCNA binding domain of p21 resulted in a more robust G1 arrest that was comparable to the expression of full-length p21, suggesting that p21 effects on PCNA are sufficient for G1 arrest in hyperoxia.

Besides regulating PCNA-dependent processivity, other studies have demonstrated that p21 also controls PCNA abundance, particularly over prolonged periods of time such as when cells enter senescence or age (11, 27). For example, PCNA expression was shown to be higher in terminally differentiated cardiomyocytes and kidneys of p21−/− mice compared with wild-type controls (11, 25). Also consistent, PCNA...
levels were shown to decline as p21 levels increased in aged chondrocytes (22). Another mechanism by which p21 might regulate PCNA is by controlling intranuclear localization of PCNA. PCNA resides in one of two nuclear pools: either a free nucleoplasmic pool or a detergent-resistant chromatin-bound fraction that is associated with sites of DNA replication or repair (4). Interestingly, high p21 expression has been shown to regulate PCNA-chromatin association (2, 5, 41). Collectively, these studies suggest that p21 can regulate PCNA at multiple levels.

Although a role for p21 in directly inhibiting PCNA-dependent DNA synthesis has been well established, it is possible that the p21-dependent regulation of PCNA abundance also contributes to growth arrest. Given that PCNA abundance can be regulated by the prolonged expression of p21 in aged or senescent cells, this study explores the possibility that the continuous expression of p21 in hyperoxia functions to lower PCNA abundance and that the loss of PCNA contributes to the activation or maintenance of growth arrest when p21 is present.

MATERIALS AND METHODS

Cell line and oxygen exposures. Human lung adenocarcinoma H1299 cells were cultured in 5% CO2 at 37°C in DMEM (high glucose) with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 200 μg/ml hygromycin. Stable clones with doxycycline-inducible expression of enhanced green fluorescent protein (EGFP) fused amino-terminal to human p21 (EGFPp21) and EGF fused amino-terminal to human p27 (EGFPp27) were created with the pBig2i expression plasmid (18). The p211-140 domain sequence was generated by RT-PCR of RNA isolated from human colon carcinoma HCT116 cells using the forward 5′-ATGT-CAGAACCACAAGG-3′ and reverse 5′-TCACCCCTGAGAG-TTCCAGGTCC-3′ primers. An EcoRI site was added to the 5′ primer, and a BamHI site was added to the 3′ primer. The EcoRI- and BamHI-digested fragment was 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. A549 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Cell lines were maintained under normoxic conditions (room air with serum, 50 U/ml penicillin, and 50 μg/ml aprotinin and 100 μg/ml phenylmethylsulfonyl fluoride). The lysate was cleared by centrifugation, and protein concentrations were determined by the Lowry Assay (DC Protein Assay; Bio-Rad, Hercules, CA). Membranes were probed with anti-p21 antibodies CP36 (1:20; kindly provided by Jiyong Zhao) and SX118 (1:500; PharMingen, San Diego, CA) or anti-EGFP (1:1,000; Clontech), anti-FLAG (1:250; Sigma, St. Louis, MO), anti-PCNA (1:1,000; Zymed, San Francisco, CA), anti-lamin B (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (1:5,000; Sigma). Antibodies were diluted in Tris-buffered saline plus 0.1% Tween 20 plus 5% milk. Membranes were then incubated in the appropriate secondary antibody before being visualized by chemiluminescence using ECL Plus (Amersham, Buckinghamshire, England). Western blots that were used for quantitative analysis were captured, and PCNA band intensities were quantified and normalized to β-actin expression using a Fluorochrome 8900 (Alpha Innotech, San Leandro, CA).

Flow cytometry. Cells were trypsinized and centrifuged at 1,200 g for 5 min. Cells were then resuspended in 75% ethanol and incubated for 24 h at 4°C. Ethanol-fixed cells were centrifuged and resuspended in 1 ml of RNase (1 mg/ml) for 30 min, centrifuged, and then resuspended in 0.5–1 ml of propidium iodide (10 μg/ml). Flow cytometry was performed, and 10,000 gated events were collected using a BD FACSCalibur System (BD Biosciences, Franklin Lakes, NJ). Cell cycle analysis was performed using ModFit LT software (Verity Software House, Topsham, ME).

PCNA immunostaining. H1299 + EGFp21 cells were plated on four-well chamber slides (BD Falcon, Bedford, MA). Cells were cultured in the absence or presence of doxycycline for 24 h and then fixed in acetone:methanol (50:50) for 2 min at room temperature. Adherent cells were blocked with 3% normal donkey serum for 30 min at room temperature, and endogenous biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA).

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**Fig. 1.** Hyperoxia stimulates p21 and inhibits PCNA expression in vivo. Adult p21+/– or p21–/– mice were exposed to normoxia (time 0) or 100% oxygen for 24, 48, and 72 h. A: Western blot analysis of PCNA, p21, and β-actin of lysates prepared from lungs of p21+/– or p21–/– mice. Images are representative of results obtained from 3 separate sets of extracts. B: densitometry was performed on 0- and 72-h samples from 3 sets of protein extracts, and band intensities were graphed (P < 0.05). PCNA expression decreased significantly at 72 h of hyperoxia only in p21+/– samples.
PCNA was detected by overnight incubation at 4°C with anti-PCNA (1:1,000, Zymed) monoclonal antibody. The slides were then incubated with a biotinylated donkey anti-mouse secondary antibody at 1:200 for 90 min at room temperature, and PCNA signal was detected by incubation with Texas red-conjugated avidin for 30 min at room temperature. Slides were then washed and immersed in 4',6-diamidino-2-phenylindole and visualized with a Nikon E800 fluorescence microscope (Nikon, Melville, NY). Expression of EGFP was directly visualized by EGFP fluorescence.

Chromatin fractionation. Chromatin-bound protein fractions were extracted essentially as previously described (32). Briefly, H1299 + EGFP21 cells were cultured in the absence or presence of doxycycline for 24, 48, or 72 h. Adherent cells were washed, trypsinized, and pelleted by centrifugation at 1,200 g for 5 min. The cells were washed twice and resuspended in 0.5 ml of lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM DTT, protease and phosphatase inhibitors). The samples were incubated on ice for 30 min and then centrifuged for 5 min at 14,000 g. The cell pellet was recovered, washed twice with lysis buffer, and then solubilized in lysis buffer containing 50 mM Tris, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM DTT, protease and phosphatase inhibitors plus 1% SDS. Nucleic acids were sheared by sonication (Heat Systems; Ultrasonics, Farmingdale, NY). Protein concentrations were quantified, and Western blotting was performed as described. For samples containing total PCNA, the cell pellet was directly solubilized in lysis buffer containing 1% SDS, and sample was treated as described above.

RNA interference treatment. H1299 + EGFP21 or A549 cells were plated in six-well plates overnight. Cells were then transfected with luciferase (Dharmacon, Lafayette, CO), PCNA (SMARTpool, Dharmacon), or p21 (SignalSilence; Cell Signaling, Danvers, MA) small interfering RNA (siRNA). p21 RNA interference (RNAi) was performed at 100 nM while the concentration of PCNA siRNA was varied between 0.25 and 50 nM. siRNA targeting luciferase was used at the highest dose of p21 or PCNA siRNA for a given experiment. Cells were transfected using Lipofectamine 2000 transfection reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). A high level of transfection efficiency was verified 12–24 h posttransfection using a fluorescently labeled siRNA (Invitrogen). For PCNA RNAi, the cells were trypsinized and replated at a lower density in 100-mm dishes and treated with medium containing or lacking doxycycline (2 μg/ml) 24 h after transfection. After an additional 24 h, cells were grown in normoxic or hyperoxic conditions for 48 h, and samples were then harvested for flow cytometry or Western blotting as described above. For p21 RNAi in A549 cells, cells were treated with hyperoxia 24 h posttransfection, and the

Fig. 2. Loss of p21 restores PCNA abundance and results in G1 checkpoint bypass and increased cell death. A549 cells were transfected with control luciferase (L) or small interfering RNA (siRNA) targeting p21 (100 nM). After 24 h, cells were grown in normoxic or hyperoxic conditions and retransfected 48 h after the original transfection. After 72 h of hyperoxic exposure, cells were harvested for flow cytometry or Western blotting. A: Western blot for PCNA, p21, or β-actin demonstrating increased expression of p21 and decreased PCNA in cells exposed to hyperoxia while siRNA silencing of p21 restored PCNA level. B: the results of 3 separate experiments were quantified and graphed. Exposure to hyperoxia and treatment with control siRNA along with hyperoxia resulted in a significant reduction in PCNA expression, whereas siRNA targeting of p21 did not significantly alter PCNA expression relative to normoxia-treated samples (*P < 0.05). C and D: DNA histograms obtained from A549 cells were quantified, and results from hyperoxia and p21 siRNA-treated samples were graphed (n = 3) (*P < 0.05). Knockdown of p21 resulted in the loss of the G1 checkpoint and the accumulation of cells in S phase as well as increased sub-G1 DNA content (values are shown).
transfection was repeated after an additional 24 h. Cells were exposed to hyperoxia for a total of 72 h.

**Statistical analysis.** Values are means ± SD. Group means were compared by ANOVA using Fisher’s procedure of post hoc analysis with Excel software (Microsoft). \( P < 0.05 \) was considered significant.

**RESULTS**

Hyperoxia stimulates p21 and inhibits PCNA expression in vivo. To determine whether p21 affects PCNA abundance during in vivo exposure to hyperoxia, p21-wild-type and p21-deficient adult mice were exposed to normoxia (time 0) or hyperoxia for 24, 48, and 72 h, and whole lung protein extracts were immunoblotted for p21, PCNA, and β-actin. Exposure to hyperoxia resulted in p21 induction and a time-dependent loss of PCNA in p21 wild-type mice, whereas PCNA levels did not decline in mice lacking p21 (Fig. 1A). Quantifying band intensities from three separate animal exposures confirmed that hyperoxia significantly reduced PCNA expression in p21 wild-type, but not in p21-deficient, mice (Fig. 1B). The loss of PCNA expression with increasing p21 during chronic exposure to hyperoxia is consistent with p21-dependent regulation of PCNA protein abundance.

**Loss of p21 restores PCNA abundance and results in G1 checkpoint bypass and increased cell death.** To determine whether p21 expression regulates PCNA abundance in a cell line model, human lung carcinoma A549 cells were treated with hyperoxia for 72 h, and samples were collected for Western blotting and flow cytometry. In this cell line, expression of p53 and p21 increase in response to hyperoxia, whereas cells arrest predominantly in G1 (35). Consistent with a role for p21 in regulating PCNA abundance, exposure to hyperoxia increased expression of p21 while decreasing PCNA (Fig. 2A). Interestingly, siRNA knockdown of p21 during hyperoxia restored PCNA abundance, whereas siRNA oligonucleotides against luciferase did not. Quantitation of PCNA Western blots obtained from three separate experiments confirmed that exposure to hyperoxia alone or treatment with control siRNA and hyperoxia resulted in a significant reduction in PCNA expression, whereas p21 knockdown did not significantly alter expression of PCNA relative to untreated cells (Fig. 2B). Further supporting a role for p21 in the activation of the G1 checkpoint in hyperoxia, p21 knockdown in A549 cells significantly reduced cells in G1 while increasing the percentage of cells in S phase (Fig. 2, C and D). In addition, treatment with p21 but not...
control siRNA resulted in increased cell death as measured by sub-G1 DNA content (Fig. 2C). These findings are consistent with a role for the p21-dependent downregulation of PCNA abundance in hyperoxia-induced growth arrest.

**EGFp21 and p21^176-164 stimulate the loss of PCNA expression.** To determine whether continuous expression of p21 could alter PCNA levels, H1299 + EGFp21 or H1299 + p21^176-164 cells were treated in the absence or presence of doxycycline for 24, 48, and 72 h, and protein extracts were immunoblotted for p21 and PCNA. Overexpression of EGFp21 or p21^176-164 resulted in a time-dependent decrease in PCNA abundance (Fig. 3, A and B). Consistent with the requirement of the carboxy-terminal PCNA binding domain to control PCNA abundance, conditional expression of a carboxy-terminal truncated form of p21 (p21^1-146) did not alter PCNA abundance (Fig. 3C). Likewise, conditional overexpression of EGFp27 (p27 fused to the carboxy terminus of EGFP) also failed to affect PCNA abundance (Fig. 3D). p27 exerts G1 growth arrest through a homologous amino-terminal cdk-binding domain but lacks the carboxy-terminal PCNA binding domain found in p21 (18). PCNA Western blots obtained from three separate experiments for each cell line were quantified and graphed. Only expression of EGFp21 and p21^176-164 resulted in a significant decrease in PCNA abundance. These data suggest that the loss of PCNA seen in cells expressing EGFp21 or p21^176-164 was not the result of G1 growth arrest but rather a specific lowering of PCNA levels by the PCNA-binding domain of p21.

**p21 acts on the total PCNA pool.** It is conceivable that the decline in PCNA level observed with p21 expression may actually represent the relocalization of PCNA to a detergent-insoluble chromatin fraction. In this case, chromatin-associated PCNA may have been lost during processing of the cell lysates. To determine whether total PCNA abundance or PCNA relocalization to chromatin was affected by EGFp21 expression, H1299 + EGFp21 cells were cultured in the absence or presence of doxycycline to induce EGFp21 expression. Cells were then fixed in acetone:methanol (50:50) and immunostained for PCNA expression to detect both chromatin-bound and soluble PCNA (1). Treatment of H1299 + EGFp21 cells with doxycycline resulted in the induction of EGFp21 as detected by EGFP fluorescence and a decrease in PCNA immunostaining (Fig. 4A). To verify that EGFp21 expression does not induce PCNA chromatin binding, H1299 + EGFp21 cells were cultured in the presence of doxycycline for 24, 48, or 72 h, and protein extracts containing total, Triton-soluble, or Triton-insoluble (chromatin-bound) proteins were harvested. Protein extracts were immunoblotted for PCNA or β-actin and were compared with samples obtained at time 0 or after 72 h of culture in the absence of doxycycline. Expression of EGFp21 resulted in the time-dependent loss of PCNA abundance in cells treated with doxycycline in all cellular-fractions (Fig. 4B). Therefore, the continued expression of p21 functions to decrease the abundance of the total PCNA pool and does not induce PCNA chromatin binding. Expression of lamin B was detected in the Triton-insoluble fraction and not the Triton-soluble fraction, demonstrating enrichment for chromatin-bound proteins. Figure 4B is representative of Western results obtained from three separate experiments.

**Loss of PCNA results in S phase arrest in normoxia.** To determine the potential cell cycle effects of p21-dependent PCNA loss, an RNAi knockdown approach was utilized. H1299 + EGFp21 cells were either untreated or transfected with control (luciferase) or increasing amounts of PCNA siRNA. Cells transfected with PCNA siRNA were grown in the absence of doxycycline to induce EGFp21 fusion protein for 24, 48, or 72 h. Total, Triton-soluble, or Triton-insoluble protein extracts were prepared as described, and extracts were immunoblotted with PCNA and lamin B or β-actin as a loading control. The result is representative of 3 separate experiments. Quantified PCNA expression is presented below each set of Western blots. DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein.

Fig. 4. p21 acts on the total PCNA pool. A: H1299 + EGFp21 cells were cultured in the absence or presence of doxycycline for 24 h and then fixed for PCNA immunostaining. Total PCNA abundance as detected by Texas red fluorescence decreased with expression of EGFp21. B: H1299 + EGFp21 cells were cultured in the absence (−) or presence (+) of doxycycline to induce EGFp21 fusion protein for 24, 48, or 72 h. Total, Triton-soluble, or Triton-insoluble protein extracts were prepared as described, and extracts were immunoblotted with PCNA and lamin B or β-actin as a loading control. The result is representative of 3 separate experiments. Quantified PCNA expression is presented below each set of Western blots. DAPI, 4′,6-diamidino-2-phenylindole; EGFP, enhanced green fluorescent protein.
either fixed for flow cytometry or protein lysates were obtained for Western blotting. Silencing of PCNA by siRNA resulted in a dose-dependent loss of PCNA protein abundance compared with control treated samples (Fig. 5A). Interestingly, while expression of EGFP21 and PCNA siRNA resulted in similar lowering of PCNA level, EGFP21 expression resulted in G1 arrest, whereas siRNA treatment caused an increase in S phase accumulation (Fig. 5B). DNA histograms from cells treated with doxycycline or PCNA siRNA (10 nM) were quantified and graphed. While doxycycline treatment activated a G1 checkpoint, siRNA knockdown of PCNA significantly increased the percentage of cells in S phase relative to untreated cells (Fig. 5C). These results demonstrate that the level of PCNA loss obtained with PCNA siRNA or EGFP21 expression is insufficient to elicit G1 arrest under normoxic conditions, and concomitant expression of EGFP21 is required for G1 arrest. In addition, small amounts of functional PCNA are sufficient to allow S phase entry, but higher PCNA levels are required to complete DNA synthesis. Treatment with luciferase (control) siRNA did not affect PCNA abundance or cell cycle distribution.

Exposure to hyperoxia downregulates PCNA abundance. To determine whether hyperoxia alone affected expression of PCNA, H1299 + EGFP21 cells were treated in the absence or presence of doxycycline and exposed to hyperoxia for 24, 48, or 72 h. Western blotting for PCNA, p21, or β-actin was performed on protein extracts collected at each time point. Interestingly, exposure to hyperoxia alone resulted in a time-dependent loss of PCNA, whereas expression of EGFP21 accelerated this loss (Fig. 6). The result shown is representative of three separate experiments.

Loss of PCNA functions in the activation of the G1 checkpoint in hyperoxia. Although PCNA lowering alone was insufficient to induce G1 arrest under normoxic conditions, it is conceivable that the p21-dependent loss of PCNA in hyperoxia could contribute to G1 arrest since PCNA abundance is additionally lowered by hyperoxia. H1299 + EGFP21 cells were treated with control or PCNA siRNA as in Fig. 5 and then

Fig. 5. Loss of PCNA results in S phase arrest in normoxia. H1299 + EGFP21 cells were transfected with control or PCNA siRNA for 24 h, treated in the absence of doxycycline for 24 h, and then grown under normoxic conditions for 48 h. For comparison, untransfected cells were treated in the absence or presence of doxycycline and grown under normoxic conditions. A: Western blot analysis for PCNA, p21, or β-actin demonstrating dose-dependent knockdown of PCNA by siRNA. Quantified PCNA expression is shown below Western blot. B: DNA histograms of H1299 + EGFP21 cells treated with PCNA siRNA demonstrating a dose-dependent S phase accumulation relative to control treated cells. C: DNA histograms obtained from H1299 + EGFP21 cells treated with doxycycline or transfected with PCNA siRNA (10 nM) were graphed and compared with untreated cells (n = 3). *P < 0.05.

Fig. 6. Exposure to hyperoxia downregulates PCNA abundance. H1299 + EGFP21 cells were treated in the absence or presence of doxycycline and exposed to hyperoxia for 24, 48, or 72 h. Cell lysates were prepared and immunoblotted for PCNA, p21, and β-actin. The result is representative of 3 separate experiments. PCNA abundance was quantified and normalized to β-actin, and average values are depicted below each lane.
exposed to hyperoxia for 48 h. Untransfected cells were grown in the absence or presence of doxycycline and treated with hyperoxia for 48 h. Titration of PCNA level by siRNA resulted in lowering of PCNA to levels comparable to those seen with EGFp21 expression (Fig. 7A). Western blots were exposed longer to account for the loss of PCNA seen with hyperoxia. Interestingly, siRNA knockdown of PCNA in hyperoxia resulted in a partial restoration of the G1 checkpoint (Fig. 7B). DNA histograms obtained from cells treated in the absence or presence of doxycycline or PCNA siRNA (10 nM) were quantified and graphed. While doxycycline treatment activated a robust G1 checkpoint, knockdown of PCNA also resulted in a significant restoration of the G1 checkpoint and decreased the percentage of cells arrested in S phase (Fig. 7C). Knockdown of PCNA in hyperoxia to a similar amount seen with EGFp21 expression is therefore sufficient to partially restore G1 arrest in the absence of p21 expression.

PCNA downregulation potentiates p21-mediated G1 arrest in hyperoxia. Since PCNA is an abundant protein and p21 is not, the loss of PCNA might allow the rate-limiting amount of p21 to be more effective at inhibiting PCNA functions, thereby exerting G1 arrest. To test this, H1299 + EGFp21 cells were transfected with siRNA against PCNA, treated with doxycycline to induce p21, and exposed to hyperoxia for 48 h. As expected, further knockdown of PCNA by siRNA in doxycycline-treated H1299 + EGFp21 cells significantly potentiated p21-dependent G1 growth arrest in hyperoxia (Fig. 8, A and B).

DISCUSSION

As with other forms of genotoxic stress, exposure to hyperoxia results in the activation of cell cycle checkpoints (29). G1 checkpoint activation is mediated in part by p21-dependent functional inhibition of G1 cdks and PCNA (6, 23). Additionally, p21 can regulate PCNA protein abundance; however, it is not known whether the loss of PCNA functions in the activation of the checkpoints in response to damage (12). In this study, we found that the prolonged expression of p21 in cell lines or mice resulted in the time-dependent loss of PCNA. Knockdown of endogenous p21 in A549 cells prevented the p21-dependent loss of PCNA in hyperoxia, resulting in S phase accumulation and cell death. The p21-dependent loss of PCNA was dependent on expression of the carboxy-terminal PCNA binding domain of p21 and was also not due to PCNA relocalization to chromatin. By carefully titrating PCNA level using RNAi, the loss of PCNA obtained by expression of p21 in H1299 + EGFp21 cells was approximated. Although the loss of PCNA abundance was insufficient to activate the G1 checkpoint under normoxic conditions, treatment with hyperoxia and PCNA siRNA resulted in further lowering of PCNA and a partial restoration of the G1 checkpoint. In addition, PCNA knockdown in doxycycline-treated H1299 + EGFp21 cells exposed to hyperoxia significantly augmented p21-mediated growth arrest in G1. These findings establish that continuous p21 expression functions to decrease PCNA abundance to potentiate p21-mediated G1 arrest.

Several lines of evidence were used to establish that p21 regulates PCNA abundance in response to hyperoxia. Expression of p21 was associated with the loss of PCNA in vivo and in cell line models. On the other hand, PCNA abundance did not change with expression of EGFp27, which does not bind PCNA but has been previously shown to induce a robust G1 checkpoint by inhibiting cyclin/cdk activity (18). The loss of PCNA downregulation potentiates p21-mediated G1 arrest in hyperoxia. Since PCNA is an abundant protein and p21 is not, the loss of PCNA might allow the rate-limiting amount of p21 to be more effective at inhibiting PCNA functions, thereby exerting G1 arrest. To test this, H1299 + EGFp21 cells were transfected with control or PCNA siRNA for 24 h, treated in the absence of doxycycline for 24 h, and then exposed to hyperoxia for 48 h. For comparison, untransfected cells were treated in the absence or presence of doxycycline and grown in hyperoxia for 48 h. A: Western blot analysis of hyperoxic treated cells for PCNA, p21, or β-actin demonstrating dose-dependent knockdown of PCNA by siRNA. Quantitated PCNA expression is presented below Western blot. B: DNA histograms of H1299 + EGFp21 cells treated with PCNA siRNA demonstrating a dose-dependent G1 accumulation relative to control treated cells. C: DNA histograms obtained from H1299 + EGFp21 cells treated with doxycycline or transfected with PCNA siRNA (10 nM) were graphed and compared with untreated cells (n = 3). *P < 0.05.
PCNA abundance observed with continuous expression of p21 is therefore a specific mechanism to decrease PCNA and not the result of cells being arrested in G1. Genetic approaches used in this study also define a role for the loss of PCNA abundance observed with continuous expression of p21 affecting PCNA protein stability (data not shown). In addition, given that PCNA abundance declined with EGFp21 and p21 expression but not expression of p21 or EGFp27, it is likely that a p21/PCNA interaction is involved in the observed loss of PCNA. The continuous production of p21 over days of hyperoxia would theoretically lower levels of PCNA until a threshold is reached whereby its activity could be functionally inhibited.

PCNA knockdown resulted in G1 accumulation only during hyperoxia, suggesting that G1 growth arrest through PCNA loss may be a damage-specific response. Surprisingly, PCNA expression was decreased by hyperoxia in the absence of p21 expression. Therefore, p21-independent signals may also lower PCNA in response to hyperoxia, particularly in p53-deficient cells since p53 can positively regulate PCNA expression (26). It is possible that exposure to hyperoxia results in oxidative damage to PCNA which is then targeted for degradation. Indeed, it has been demonstrated that oxidized proteins are preferentially degraded by the proteasome (10). In addition, PCNA can be a target for ubiquitination and has been shown to be regulated by the proteasome (11, 20, 43). G1 arrest may be achieved in the absence of p21 inhibitory activity due to the cumulative lowering of PCNA by siRNA and hyperoxia. Alternatively, exposure to hyperoxia could directly poison or inhibit PCNA activity resulting in G1 accumulation when PCNA abundance is decreased by RNAi. Since PCNA siRNA treatment induces S phase arrest, the loss of PCNA abundance by hyperoxia may be responsible in part for the dramatic S phase arrest typically seen in p21-deficient cells exposed to hyperoxia (16).

While PCNA has previously been shown to be regulated transcriptionally and posttranscriptionally, Engel et al. (11) provide evidence that p21 enhances PCNA proteasomal degradation. Our data are consistent with this in that the loss of PCNA seen with p21 expression represents a decline in total protein abundance rather than a redistribution of PCNA within the nuclear compartment. Unfortunately, we were unable to determine if p21 expression enhanced the proteasomal degradation of PCNA as seen in cardiomyocytes due to the toxicity of treating cells with proteosome inhibitors for prolonged periods of time; however, preliminary results are consistent with p21 affecting PCNA protein stability (data not shown). In addition, given that PCNA abundance declined with EGFp21 and p21 but not expression of p21 or EGFp27, one is likely that a p21/PCNA interaction is involved in the observed loss of PCNA. The continuous production of p21 over days of hyperoxia would theoretically lower levels of PCNA until a threshold is reached whereby its activity could be functionally inhibited.

The chronic production of ROS during hyperoxia has been argued to model the effects of aging (13, 40). If so, our findings help clarify why PCNA levels decline during cellular senescence since p21 abundance has been shown to increase in aged or senescent cells (24, 28, 37, 38). Thus it is conceivable that the p21-dependent lowering of PCNA level to induce G1 arrest in response to chronic exposure to hyperoxia may model, on a compressed time scale, the long-term inhibition of cell division by p21 in differentiated or aged tissue. Additionally, the age-dependent decline in PCNA protein abundance has been associated with decreased DNA repair capacity, and the PCNA binding domain of p21 was shown to inhibit DNA repair (8, 14, 33). Therefore, p21 could act to lower PCNA abundance to prevent repair under conditions of prolonged oxidative stress as seen in aged cells. Failure to prevent abortive repair leads to mutations that may contribute to cancer or cell death, processes that are widely observed as cells age.

Although our data demonstrate that the prolonged expression of p21 during hyperoxia functions to lower PCNA abundance to exert G1 arrest, there are limitations to this study. First, it was not possible to completely separate p21-mediated cell cycle effects resulting from the inhibition of PCNA activity from those resulting from the loss of PCNA abundance. To circumvent this issue, we titrated PCNA knockdown using siRNA to approximate the level seen with EGFp21 expression in H1299 cells, therefore allowing us to determine the potential cell cycle effects of PCNA loss without the confounding effects of p21 inhibitory activity. In addition, due to the prolonged nature of the observed effects, we were not able to fully address the mechanism by which p21 decreases PCNA abundance in hyperoxia.

The loss of p21 in A549 cells exposed to hyperoxia resulted in a partial loss of the G1 checkpoint and increased cell death, suggesting that tight regulation of cell cycle progression during chronic damaging conditions is critical for survival. This study provides evidence that the p21-dependent loss of PCNA abundance during exposure to hyperoxia functions to potentiate p21 inhibitory activity and ensure G1 arrest in response to chronic oxidative challenges. Given the importance of PCNA to also control DNA repair, the persistent loss of PCNA over time may be critically important for preventing abortive DNA repair under adverse environmental conditions that might compromise the repair process. Understanding how PCNA functions
in oxidized cells could provide new therapeutic opportunities for preventing hyperoxia-induced lung injury and perhaps other tissue injuries caused by the persistent elevation of ROS.

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