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p21$^{Cip1/WAF1/Sdi1}$ Does Not Affect Expression of Base Excision DNA Repair Enzymes During Chronic Oxidative Stress

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ABSTRACT

Exposure to chronic oxidative stress during elevated oxygen (hyperoxia) damages DNA and inhibits cell proliferation in G_1 through induction of the cyclin-dependent kinase inhibitor p21. Cells that fail to express p21 growth-arrest in S phase. The observation that growth arrest in G_1 is associated with reduced DNA damage and enhanced survival suggests that p21 may affect expression of base excision repair (BER) enzymes used to repair oxidized DNA. This hypothesis was tested in *p21* wild-type and *p21*-deficient mice and human lung adenocarcinoma H1299 cells with tetracycline-on regulated expression of p21. The mRNA levels of Ogg1, Tdg, Udg, Mpg, Nth1, and Mgmt remained constant during 3 days of hyperoxia. The expression of Ogg1, Nth1, and APE protein also remained unchanged. Although hyperoxia increased p21, its absence did not significantly affect expression of these repair enzymes. These findings reveal that hyperoxia induces p21 without significantly altering BER enzyme expression. This suggests that p21 may protect oxidized cells by affecting the activity of BER enzymes and/or through other mechanisms, such as apoptosis. *Antioxid. Redox Signal.* 7, 719–725.

INTRODUCTION

Reactive oxygen species (ROS) are generated during normal aerobic respiration, during exposure to high oxygen (hyperoxia), and when neutrophils become activated. Free radical attacks on DNA produce oxidized nucleotides and sugars, depurination, depyrimidation, and single- and double-strand breaks (6). Failure to prevent or remove these lesions can be mutagenic and detrimental to survival. Several mechanisms have evolved to protect cells against the genotoxic effects of ROS, including the production of antioxidant enzymes to detoxify ROS, activation of DNA replication and repair processes that prevent fixation of mutations, and activation of apoptotic processes designed to eliminate severely damaged cells from tissues. The observation that some proteins that participate in DNA repair also inhibit DNA replication suggests that these two processes may be tightly integrated (37).

Base excision repair (BER) is the predominant mechanism to repair oxidized and fragmented DNA. Lesion-specific monofunctional glycosylases, including 8-oxoguanine DNA glycosylase-1 (Ogg1), thymidine DNA glycosylase (Tdg), and O^6-methylguanine DNA methyltransferase (Mgmt) remove damaged bases, thereby creating an abasic site (32). Apurinic/apyrimidinic endonuclease (APE) cleaves 5′ to the abasic site, resulting in a break in the phosphodiester backbone that is then repaired by DNA polymerase β and ligase. Although less understood, alterations in BER enzyme expression and/or activity have been reported when cells are damaged. For example, asbestos, ionizing radiation, sodium hypochlorite, or hydrogen peroxide increase APE expression (9, 24). Whereas genetic overexpression of APE did not significantly affect survival of Chinese hamster ovary (CHO) cells exposed to hydrogen peroxide (23), antisense oligonucleotides against APE significantly reduced clonogenic survival of oxidized HeLa cells (31). Another study showed that overexpression of Ogg1 or formamidopyrimidine DNA glycosylase (Fpg) modestly enhanced DNA integrity and survival of A549 cells exposed to hyperoxia (33). These observations reveal that alterations in BER enzymes can affect DNA integrity and therefore overall cell survival.

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In response to unfavorable environmental conditions, the tumor suppressor protein p53 accumulates and exerts G_1 growth arrest by increasing transcription of the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1/Sdi1}$ (p21) (7). Increased levels of p21 exert G_1 arrest by blocking cyclin E/cdk2 kinase and proliferating cell nuclear antigen (PCNA)-dependent DNA elongation (30). Cells that fail to express p21 exit G_1, progress slowly through S phase, and accumulate in G_2 phase. Cell-cycle checkpoints are thought to allow additional time for DNA repair or apoptosis to occur. Consistent with the hypothesis that growth arrest in G_1 favors survival, *p21*-deficient adult and newborn mice are markedly more sensitive to ionizing radiation and hyperoxia than wild-type mice (5, 22). Cell lines that fail to express p21, such as HCT116 *p21* (−/−) colon carcinoma cells or *p53*-deficient H1299 human lung adenocarcinoma cells, exhibit increased sensitivity to nitrogen mustard, cisplatin, UV radiation, and hyperoxia relative to parental cells that express p21 (8, 15, 20). p21 may protect cells from oxidative damage simply by exerting G_1 arrest where BER enzyme expression or activity may be elevated (16). Indeed, Mv1Lu cells that are arrested in G_1 by contact inhibition, treatment with transforming growth factor-β, or serum deprivation exhibit increased cell survival and reduced DNA damage when exposed to hyperoxia (25). An alternative hypothesis is that p21 may inhibit BER enzyme expression under conditions where DNA polymerase activity is repressed so that abortive DNA repair does not occur (2). In this scenario, intra-S phase arrested cells that cannot complete BER due to inhibition of DNA polymerase activity would exhibit more damage and sensitivity to hyperoxia than cells in G_1 that cannot initiate BER due to repression of BER enzyme levels.

Although the role of p21 to inhibit DNA replication and enhance survival of oxidized cells is known, it remains unclear whether it affects expression of DNA repair enzymes. The chronic nature of hyperoxia offers a unique opportunity to investigate temporal changes in expression of BER enzymes that may not be readily detected in acute models, such as that caused by ionizing radiation or hydrogen peroxide. In the current study, mice lacking p21 and human lung epithelial cell lines in which p21 expression is restored under tetracycline-dependent regulation are used to investigate whether expression of several key BER enzymes is affected by hyperoxia and p21 expression.

MATERIALS AND METHODS

Mice

C57Bl/129J outbred *p21* (−/−) mice were backcrossed 10 generations to C57Bl/6J mice obtained from Jackson Laboratories (Bar Harbor, ME, U.S.A.) and exposed to hyperoxia as previously described (22). As assessed by pulmonary compliance, edema, and overall survival, the inbred C57Bl/6J *p21*-deficient mice exhibited the same hypersensitivity to hyperoxia as the outbred line of mice described previously (5). Mice were killed with pentobarbital (65 mg/kg injected intraperitoneally). The lungs were exposed, and the left lobe used to isolate RNA and the right lobes used to isolate protein. The University of Rochester's University Committee on Animal Resources approved all exposures and handling of the mice.

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H1299 cell line

The *p53*-deficient human lung adenocarcinoma H1299 cells with tetracycline-regulated expression of p21 fused to enhanced green fluorescence protein (EGFP) were treated with doxycycline and hyperoxia (16). In the absence of doxycycline, these cells fail to induce p21 and therefore growth-arrest in S phase when exposed to hyperoxia. The induction of EGFp21 by doxycycline restores G_1 growth arrest. The box was flooded with 95% O_2/5% CO_2 for 10 min at a flow rate of 10 L/min before sealing and incubating at 37°C.

RNase protection analysis

Lungs or cells were lysed in 4 *M* guanidine isothiocyanate, 0.5% *N*-laurylsarcosine, 20 m*M* sodium citrate, and 0.1 *M* β-mercaptoethanol. RNA was extracted using acid phenol and phase-lock gel columns (5 Prime-3 Prime, Boulder, CO, U.S.A.) and resuspended in diethylpyrocarbonate-treated water. RNase protection assays were performed with the human and mouse BER-1 multiprobe template kit according to the manufacturer's instructions (PharMingen, San Diego, CA, U.S.A.). Riboprobe synthesis reaction was incubated for 60 min at room temperature, followed by an additional 30 min at 37°C in the presence of 2 units of RNase-free DNase. Riboprobes were extracted with phenol/chloroform and precipitated. Probes were resuspended in 50 μl of hybridization buffer (400 m*M* NaCl, 40 m*M* PIPES, pH 6.7, 1 m*M* EDTA, pH 8.0, and 80% formamide) and diluted to 3.37×10^5 cpm/μl before incubation with 5 μg of denatured total RNA at 56°C for 16 h. Hybridized probes were digested with RNase buffer for 45 min at 30°C. Samples were incubated with proteinase K and yeast tRNA before extraction with phenol/chloroform and precipitation with ethanol. Protected products were separated on a 6% acrylamide/8 *M* urea sequencing gel and visualized by exposure on PhosphorImager screens.

Western blot analysis

Lungs or cells were homogenized in lysis buffer containing protease inhibitors as previously described (21). Lysates were cleared by centrifugation and protein concentrations determined by the Lowry Assay (DC Protein Assay, Bio-Rad, Hercules, CA, U.S.A.). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, U.S.A.). Blocked membranes were incubated in primary antibody against Ogg1, *Escherichia coli* homologue III endonuclease (Nth1), or APE (Novus Biologicals, Littleton, CO, U.S.A.), washed in phosphate-buffered saline, and incubated in horseradish peroxidase-conjugated secondary antibody (Jackson Labs, West Grove, PA, U.S.A.). Antibody interactions were visualized by chemiluminescence (Amersham, Arlington Heights, IL, U.S.A.). Images were captured and band intensities quantified with a FluorChem 8900 gel documentation system (Alpha Innotech, San Leandro, CA, U.S.A.). As a loading control, membranes were also blotted for β-actin (Sigma, St. Louis, MO, U.S.A.).

Statistical analyses

Values are expressed as means ± SD. Group means were compared by ANOVA with Fisher's procedure *post hoc* analysis using Excel software (Microsoft Corp., Seattle, WA, U.S.A.) with $p < 0.05$ considered significant.

RESULTS

BER enzyme expression in mice exposed to hyperoxia

Total RNA was isolated from lungs of *p21* wild-type and *p21*-deficient mice exposed to room air or hyperoxia for 24, 48, or 72 h, and hybridized to radiolabeled antisense RNA probes corresponding to Ogg1, Tdg, APE, uracil DNA glycosylase (Udg), 3-methyladenine DNA glycosylase (Mpg), (Nth1), Mgmt, the ribosomal protein L32, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Protected products were resolved by denaturing electrophoresis and quantified by PhosphorImage analysis. mRNA for each of these genes was detected in lung samples prepared from mice exposed to room air, with Ogg1, Tdg, APE, and Mpg being more abundant than Udg, Nth1, or Mgmt (Fig. 1A). The mRNA expression of the ribosomal protein L32 was used to normalize changes in gene expression. With the exception of Mgmt, which increased 40%, BER mRNA was unaffected by 24, 48, or 72 h of hyperoxia (Fig. 1B). In addition, BER mRNA expression was not different in *p21*-deficient mice exposed to room air or hyperoxia (Fig. 1C).

The protein levels of DNA glycosylases Ogg1 and Nth1 were investigated because they remove oxidized purines and pyrimidines. Relative to expression of β-actin, hyperoxia increased expression of p21 without significantly altering expression of Ogg1, Nth1, or APE (Fig. 2). Closer examination revealed that hyperoxia induced proteolysis of Ogg1 after 72 h, resulting in a smaller band of ~27 kDa. Although the abundance of Ogg1, Nth1, and APE remained constant in *p21* (−/−) mice exposed to hyperoxia, proteolysis of Ogg1 was still detected after 72 h. Taken together, these findings indicate that BER enzyme expression remains fairly constant during hyperoxia and is unaffected by the absence of p21.

Regulated induction of p21 does not affect expression of BER enzymes

Previous studies showed that human lung adenocarcinoma H1299 cells arrest in S phase during hyperoxia because they lack p53 and therefore cannot induce p21 (16). Growth arrest in G_1 was restored by overexpression of p21 or the related cyclin-dependent kinase p27 fused to the EGFP. This cell line model was used to determine whether restoration of p21 and G_1 growth arrest altered expression of BER enzymes. H1299/EGFp21 cells were treated in the absence or presence of doxycycline for 24 h before exposure to room air or hyperoxia for 24, 48, and 72 h. RNase protection analysis revealed that hyperoxia did not at any time significantly alter expression of Ogg1, Tdg, APE, Udg, Mpg, Nth1, replication protein A homologue (RPA4), Mgmt, or methyl CpG binding protein 4 (Mbd4) (Fig. 3A and B). A small, but significant, increase in single-strand monofunctional Udg (Smug1) was detected. The relative abundance of each enzyme was strikingly different between H1299 cells and mice. For example, Udg was readily detected in H1299 cells, but not in mice, whereas

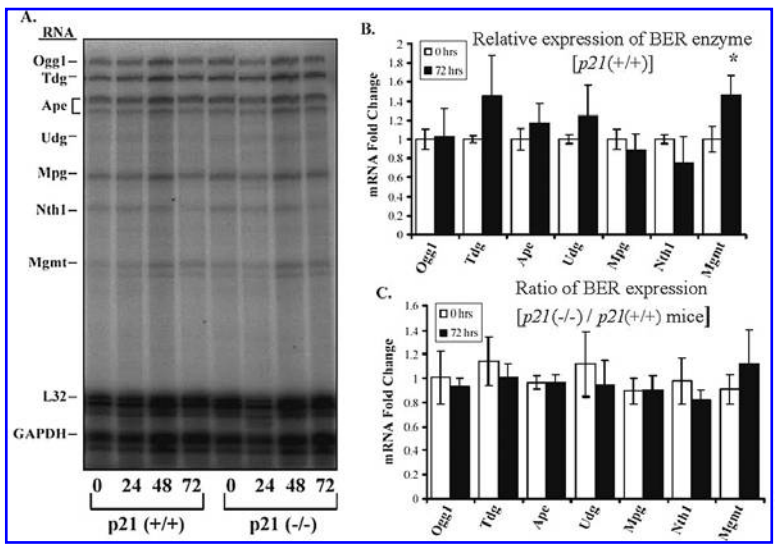

FIG. 1. Hyperoxia does not alter mRNA expression of BER genes in mice. *p21* (+/+) and *p21* (−/−) mice were exposed to room air (0) or hyperoxia for 24, 48, and 72 h. **(A)** Representative RNase protection assay using 5 μg of total lung RNA. **(B)** Protected products in *p21* (+/+) mice exposed to room air (open bars) and 72 h of hyperoxia (filled bars) were normalized to L32 and graphed relative to room air values. **(C)** The values obtained from *p21* (−/−) mice were divided by the values obtained from *p21* (+/+) mice exposed to room air (open bars) or 72 h of hyperoxia (filled bars). Values above 1 indicate that expression was higher in *p21* (−/−) lungs, whereas values below 1 indicate that expression was higher in *p21* (+/+) lungs. Bars in B and C represent means ± SD ($n = 3$ where $*p < 0.05$).

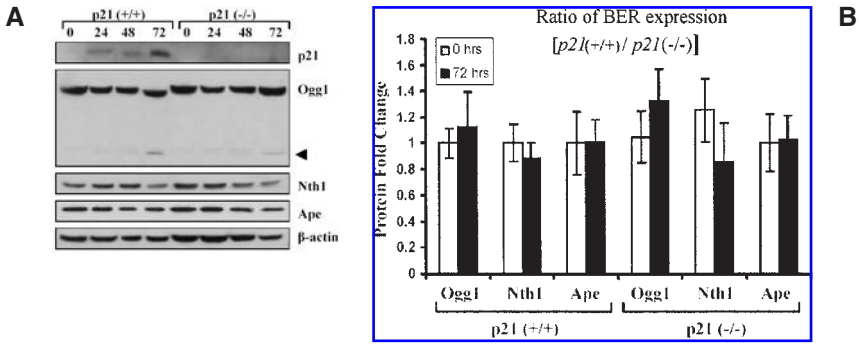

FIG. 2. Hyperoxia does not alter BER enzyme expression in mice. *p21* (+/+) and *p21* (−/−) mice were exposed to room air or hyperoxia for 24, 48, and 72 h. **(A)** Representative western blot of lung (10 μg) immunoblotted for Ogg1, Nth1, APE, p21, and β-actin. Arrow points to a smaller proteolytic fragment of Ogg1. **(B)** The values obtained from *p21* (−/−) mice were divided by the values obtained from *p21* (+/+) mice exposed to room air or 72 h of hyperoxia (filled bars). Bars represent means ± SD ($n = 3$ where $*p < 0.05$).

Mgmt was not readily detected in H1299 cells, but was in mice. Overexpression of EGFp21 did not affect mRNA expression of any enzyme except Mgmt (Fig. 3C). However, the significance of this change is unclear because Mgmt expression was barely detectable above background and required long exposures. BER mRNA expression was also unaffected when cells were arrested in G_1 by expression of EGFp27 or increased cell density (data not shown). Genetic deletion of p21 in human colon HCT116 cells, which led to an S-phase arrest during hyperoxia, also did not affect BER expression during hyperoxia (15). Taken together, these observations reveal that hyperoxia and cell-cycle arrest in G_1 do not markedly affect basal mRNA levels of BER enzymes in H1299 cells.

Western blot analysis was used to investigate whether protein levels were altered. Like in the mice, expression of Ogg1, Nth1, and APE remained uniform throughout the exposure to hyperoxia (Fig. 4A). Although high levels of EGFp21 were obtained when cells were cultured in doxycycline, this did not alter abundance of BER enzymes. Proteolysis of Ogg1 during hyperoxia was not observed in these cells. Similar findings were observed in H1299 cells arrested in G_1 by overexpression of EGFp27 or increased cell density, as well as in HCT116 cells where p21 was deleted (data not shown). These

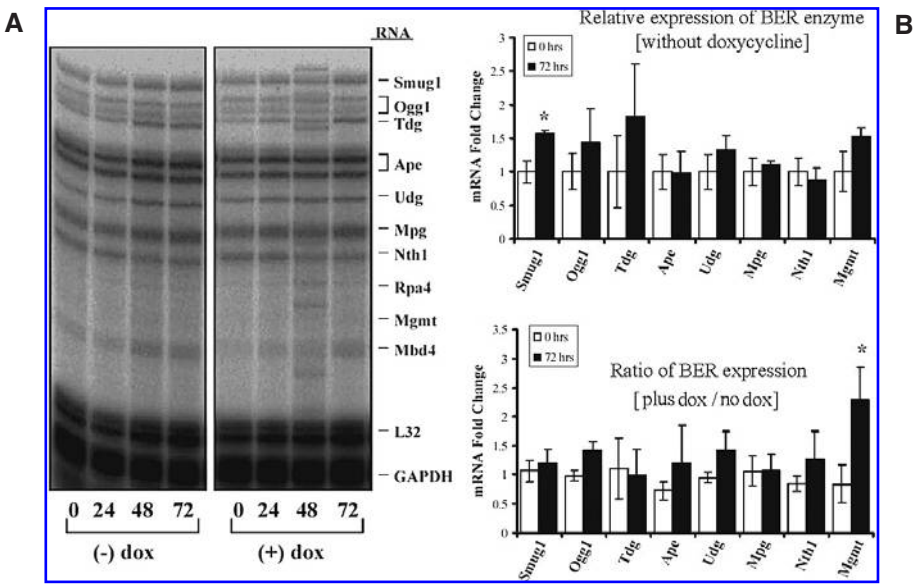

FIG. 3. Hyperoxia does not alter mRNA expression of BER genes in H1299 cells. H1299/EGFp21 cells were treated with or without 2 μg/ml doxycycline for 24 h before exposure to room air (0) or hyperoxia for 24, 48, and 72 h. **(A)** Representative RNase protection assay using 5 μg of total RNA isolated from cells. **(B)** Protected products in untreated cells were normalized to L32 and graphed relative to room air values. **(C)** The values obtained from H1299 cells treated with doxycycline were divided by the values obtained from H1299 cells. Bars in B and C represent means ± SD ($n = 3$ where $*p < 0.05$).

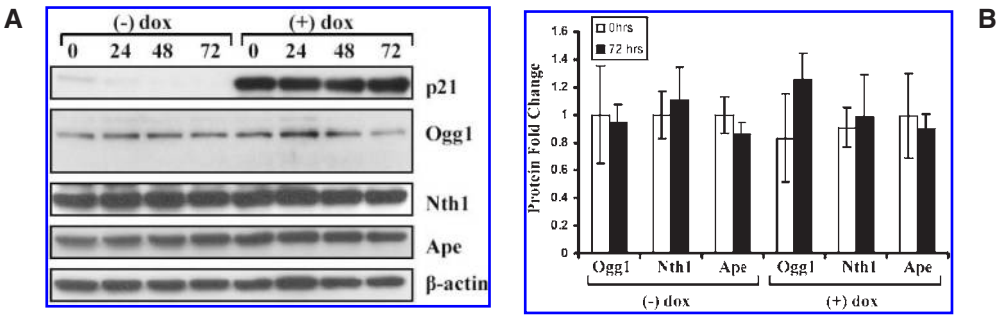

FIG. 4. Hyperoxia does not alter BER enzyme expression in H1299 cells. H1299/EGFp21 cells were treated in the presence and absence of 2 μg/ml doxycycline for 24 h before exposure to room air (0) or hyperoxia for 24, 48, and 72 h. **(A)** Representative western blot of cell lysates (10 μg) immunoblotted for Ogg1, Nth1, APE, EGFp21, and β-actin. **(B)** The values obtained from H1299 cells treated with doxycycline were divided by the values obtained from H1299 cells. Bars represent mean ± SD ($n = 3$ where $*p < 0.05$).

findings reveal that growth arrest in G_1 did not alter expression of many BER enzymes during hyperoxia.

DISCUSSION

This study provided evidence that BER enzyme expression is not markedly affected by chronic oxidative stress caused by hyperoxia. Moreover, BER enzyme expression was unaffected by the absence or presence of p21. The lack of gene changes during chronic oxidative stress are consistent with another study showing that acute oxidative damage caused by hydrogen peroxide, paraquat, or ionizing radiation does not affect expression of fpg, mutY, nei, or nth DNA glycosylases in *E. coli* (11). It is also consistent with a recent gene array study in *S. cerevisiae* showing that gene expression profiles do not correlate with survival against a wide variety of genotoxic agents (1). The observation that DNA repair enzymes are very abundant suggests that cells may have evolved posttranscriptional mechanisms to regulate DNA repair rather than relying on damaged DNA to faithfully transcribe repair enzymes. Indeed, proteolysis of Ogg1 was detected in *p21* wild-type and *p21*-deficient mice exposed to hyperoxia. Although the significance remains unclear, a smaller 32-kDa fragment has been reported in livers of mice treated with the carcinogen 3′-methyl-4-dimethylaminoazobenzene (17). In that study, the authors speculate that proteolysis might reduce repair capacity, which might account for why 8-oxoguanine lesions accumulate during hyperoxia (26).

An important observation in this study is that the expression of many BER enzymes remains fairly constant during hyperoxia. This is different from other studies showing that APE is modestly regulated at the transcriptional level by asbestos, hydrogen peroxide, NaOCl, or HOCl (9, 24). Increased APE expression was associated with enhanced DNA repair and adaptation to subsequent ROS challenge. APE levels also increased in HT29 cells exposed to hypoxia and persisted when cells were returned to a normoxic environment (36). As hypoxia is not genotoxic, the induction of APE may be more important for its ability to restore transcriptional activity to oxidized transcription factors such as activator protein-1 (AP-1), nuclear factor-κB (NF-κB), and p53 (18, 34). Indeed, APE and redox factor (Ref)-1 are the same protein whose ability to repair DNA and reduce oxidized proteins is encoded by separate domains (35). Thus, not only does APE/Ref-1 stimulate DNA repair, it can also stimulate p53 transcriptional activity toward its target genes (10). APE also reduces oxidized AP-1, NF-κB, and hypoxia-inducible factor-1α, suggesting a link exists between transcription factor signaling and DNA repair (37). Intriguingly, overexpression of APE enhanced sensitivity of CHO cells to drugs that require bioreduction to be toxic (23). Mutational analysis revealed that this effect was due to the redox, but not the DNA repair domains of APE. The observation that overexpression of APE/Ref-1 is not always protective suggests that endogenous expression must be tightly regulated under different environmental conditions, including hyperoxia.

The expression of some DNA repair enzymes has been linked to the cell cycle. Studies with synchronized HeLa cells revealed that Nth1 expression increases during S phase, whereas expression of Ogg1 remains constant throughout the cell cycle (19). The observation that mice lacking Ogg1 and Nth1 are phenotypically normal led to the identification of two additional glycosylases NEIL1 and NEIL2, so named for their homology to *E. coli* MutM/Nei genes (14). Like Nth1, NEIL1 expression is strongly increased in S phase, whereas NEIL1 remains constant throughout the cell cycle (12, 13). These findings suggest that Nth1 and NEIL1 may be important during transcription-coupled repair, whereas Ogg1 and NEIL2 are critical for genome repair. Based on the strong cell-cycle dependence of some glycosylases, it was surprising to discover that *p21* deficiency and growth arrest in S phase during hyperoxia did not affect BER enzyme levels. Although unproven, the normal induction of Nth1 and NEIL1 as cells enter S phase may be compromised by the chronic oxidative nature of hyperoxia, which is known to disrupt both transcription and translation (27).

Although G_1 growth arrest did not affect expression of DNA repair enzyme levels during hyperoxia, p21 may still participate in long-patch BER or nucleotide excision repair (NER) through its interactions with PCNA, a component of both processes. Unfortunately, it remains controversial whether p21 aids or represses DNA repair. For example, it is well-

established that *p21*-deficient mice and cell lines are acutely sensitive to ionizing radiation, cisplatin, nitrogen mustard, UV, and hyperoxia-induced genotoxic stress (8, 15, 20, 22). Although this suggests p21 enhances DNA repair, a gene chip study using HT1080 fibrosarcoma cells found that regulated overexpression of p21 led to repressed expression of several DNA repair enzymes involved in NER (3). This is consistent with other studies showing that the PCNA binding domain of p21 blocked NER in cultured cells (4, 28). Several other proteins, including flap endonuclease-1 (FEN-1) and XPG (xeroderma pigmentosum type G), compete for the p21 binding site on PCNA. PCNA stimulates BER through stabilizing FEN-1 on DNA, where it can excise flap structures (29). Interestingly, mutants of PCNA that cannot bind FEN-1 are unable to stimulate excision repair, suggesting that p21 may prevent repair by blocking association of PCNA and FEN-1. Clearly, more research needs to be done to clarify how p21 affects DNA repair.

The present study has several limitations. It remains possible that hyperoxia or p21dependent growth arrest may affect expression of BER enzymes that were not investigated. A second limitation is that subtle changes in gene expression by some cells can often be missed when gene expression is analyzed in whole lung, which contains 40 different cell types. As immunohistochemical staining of mouse lungs failed to detect significant differences in Nth1 or APE expression during hyperoxia, further studies were not performed. The use of cell lines with unknown molecular changes is another limitation of the current study. Although H1299 cells are known to lack p53, which could affect BER enzyme expression or activity, they accumulate in S phase when exposed to hyperoxia and G_1 when EGFp21 or EGFp27 is overexpressed. Although p21 can stimulate G_1 arrest during hyperoxia, the current study provides evidence that it does not significantly affect BER enzyme levels.

In summary, the expression of many BER enzymes was found to remain constant during chronic oxidative stress caused by hyperoxia. These findings are consistent with recent observations that expression of BER enzymes does not correlate with survival against genotoxic stress (1). Because aerobic respiration produces lesions that are continuously being replaced, cells may have evolved posttranscriptional mechanisms to control DNA repair rather than relying on the integrity of potentially damaged DNA to faithfully transcribe critically important repair genes. This concept is presently under investigation.

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ABBREVIATIONS

AP-1, activator protein-1; APE, apurinic/apyrimidinic endonuclease; BER, base excision repair; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescence protein; FEN-1, flap endonuclease-1; Fpg, formamidopyrimidine DNA glycosylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mbd4, methyl CpG binding protein 4; Mgmt, O^6-methylguanine DNA methyltransferase; Mpg, 3-methyladenine DNA glycosylase; NER, nucleotide excision repair; NF-κB, nuclear factor-κB; Nth1, *Escherichia coli* homologue III endonuclease; Ogg1, 8-oxoguanine DNA glycosylase-1; p21, cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1/Sdi1}$; PCNA, proliferating cell nuclear antigen; Ref, redox factor; ROS, reactive oxygen species; RPA4, replication protein A homologue; Smug1, single-strand monofunctional uracil DNA glycosylase; Tdg, thymidine DNA glycosylase; Udg, uracil DNA glycosylase.

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