Activation of the G2 cell cycle checkpoint enhances survival of epithelial cells exposed to hyperoxia

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ELEVATED LEVELS OF OXYGEN (hyperoxia) are used to treat respiratory distress in newborns, children, and adults. Although the beneficial effects of hyperoxia to reduce tissue hypoxia are well known, long-term exposures cause morbidity and mortality. Pulmonary cells may be injured directly by reactive oxygen species produced during hyperoxia as well as by oxidants derived from recruited inflammatory cells. In vitro studies revealed that the toxic effects of hyperoxia include increased production of hydrogen peroxide and superoxide (34), oxidation of DNA and lipids (4), growth inhibition (26), and ultimately cell death. With the exception of the macrophage cell line 264.7, which dies by apoptosis (22), hyperoxia kills cultured cells (A549, Mv1Lu) by necrosis, as defined by failure to exclude vital dye and the absence of laddered DNA or positive TdT-UTP nick end-labeling (TUNEL) staining (14, 15, 24). Although it remains unclear how oxygen kills cells, recent studies indicate that a number of signal transduction pathways become activated during hyperoxia, including those that inhibit cell growth (19).

Cell cycle checkpoints are signal transduction pathways that are activated when DNA is damaged. Checkpoint activation is associated with growth arrest in G1 and G2, followed by DNA repair or apoptosis. Increased genomic instability and cancer development occur when checkpoints are disrupted. The p53 tumor suppressor is the major regulator of the G1 checkpoint. p53 accumulation in damaged cells and exerts a G1 arrest by transcriptionally increasing expression of the cyclin-dependent kinase inhibitor p21 (8). p21 binds and inhibits cyclin-dependent kinases that are required for DNA replication. In contrast, p53-deficient cells typically progress through S phase and arrest in G2 phase of the cell cycle. The G2 arrest is largely p53 independent and involves activation of the ataxia-telangiectasia mutant (ATM) or its homolog ATM and Rad3-related (ATR). ATM/ATR are members of the phosphoinositol 3-kinase kinase superfamily. They phosphorylate a number of downstream targets, including serine-15 on p53 and the G2 checkpoint kinases Chk1 and Chk2 (20). The Chk proteins inhibit the cdc25C phosphatase, which removes inhibitory phosphorylations on cdc2 required for mitosis. The G2 checkpoint provides the last opportunity to repair damaged DNA before mitosis. Inactivation of the G2 checkpoint by drugs, such as caffeine, sensitizes cells to genotoxic stress (16, 28). Although caffeine is likely to affect many pathways, it appears to override G2 by inhibiting ATM and ATR (3, 13, 27, 33).

Accumulating evidence suggests that hyperoxia inhibits cell proliferation through checkpoint activation. Hyperoxia inhibited proliferation of Simian virus (SV) 40-transformed rat type II epithelial cells and in
creased expression of p21 (7). Unfortunately, flow cytometry studies have not been used to confirm that these cells arrested in G1. Additional studies revealed that hyperoxia increased p53 and p21 in A549 pulmonary adenocarcinoma cells that arrested predominantly in G1 and to a lesser extent in G2 (23). In contrast, Mv1Lu cells, which fail to express p21, arrested in S and G2 when challenged with hyperoxia. Using isogenic colon cell lines lacking p53 or p21, we showed that the G1 checkpoint was mediated solely by p53-dependent induction of p21 (14). The p21-deficient colon cells were also less viable in hyperoxia compared with p21 wild-type cells. Similarly, p21-deficient mice are more sensitive to hyperoxia, as shown by increased edema and rapid mortality (21). Although the mechanism of protection remains unclear, these data indicate that growth arrest in G1 favors survival over growth arrest in S phase and G2. Hyperoxia may also activate additional checkpoints because it still inhibits growth of p53- and p21-deficient cells. The relationship between hyperoxia-induced cell proliferation and death has not been fully established. In the current study, we demonstrate that hyperoxia activates a caffeine-sensitive G2 checkpoint that is necessary for cell viability.

MATERIALS AND METHODS

Cell culture. MLE15 cells (transformed mouse lung epithelial) were obtained from Jeffrey Whitsett (University of Cincinnati, Cincinnati, OH), and A549 cells were obtained from the American Type Culture Collection (Rockville, MD). MLE15 cells were derived from transgenic mice expressing Simian large and small-T antigen under control of the human surfactant protein-C gene promoter. The type II cell characteristics of this cell line have been published previously (31). A549 cells were derived by explanting a carcinoma obtained from a Caucasian male (9). Ultrastructural and biochemical studies revealed that it contains lamellar bodies and a high percentage of disaturated fatty acids, consistent with the type II cell phenotype (17). Although the transformed nature of this line remains unknown, we have shown that A549 cells contain an intact p53-dependent G1 checkpoint activated by ionizing radiation or hyperoxia (23). MLE15 cells were incubated at 37°C in 5% CO2 in DMEM and F-12 medium with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin (GIBCO-BRL, Grand Island, NY). A549 cells were incubated in DMEM supplemented with the same serum and antibiotics. All cells were maintained in tissue culture flasks and routinely passaged every 3 days.

Cells were trypsinized, counted with a hemacytometer, and plated in 100-mm dishes at a density of 5 × 105 cells/ml overnight. Nonadherent cells were removed by washing and replating with fresh media at which point cells were cultured in normoxia (room air with 5% CO2) or hyperoxia (95% O2-5% CO2) in a Plexiglas box (Belco Glass, Vineland, NJ). The box was sealed, and gases were delivered with a PROOX model 110 (Reming Instruments, Redfield, NY) at a flow rate of 5 l/min. The cells were harvested at various times with 0.25% trypsin, counted with a hemacytometer, and stained for viability with 0.5% Trypan blue dye.

Flow cytometry. Cells were trypsinized, resuspended in their original medium, and centrifuged at 300 g. The medium was removed, and the cells were fixed in 75% ethanol for 24 h. The cells were resuspended in 1 ml RNase (1 mg/ml) for 30 min, centrifuged, and resuspended in 0.5 ml propidium iodide (10 μg/ml). The samples were analyzed on an Epics Elite ESP (Coulter Electronics, Hialeah, FL) set to collect 10,000 events.

5-Bromo-2′-deoxyuridine incorporation. Adherent cells were incubated for 1 h in prewarmed medium containing 5-bromo-2′-deoxyuridine (BrdU; 10 μM). The cells were washed two times in PBS and incubated in medium lacking BrdU. Plates were exposed to room air or hyperoxia. In some experiments, cells were treated with 1 μg/ml colcemid. The cells were harvested and fixed in 75% ethanol for a minimum of 24 h. On the day of analysis, fixed cells were treated with 3 ml of 2 N HCl in PBS containing 0.2 mg/ml pepsin for 20 min at 37°C. Cells were centrifuged and resuspended in 1 ml of PBS containing 0.2% FBS and incubated for 45 min with 10 ng anti-BrdU-FITC (Boehringer Mannheim, Indianapolis, IN) followed by RNase treatment and propidium iodide counterstaining. Measurements of red (DNA content) and green (BrdU) fluorescence were determined by flow cytometry.

DNA laddering. Genomic DNA was purified using a TACS apoptotic laddering kit and separated by size in 1.2% Trevigel (Trevigen, Gaithersburg, MD). The gels were stained with 1:10,000 dilution of SYBR green I (Molecular Probes, Eugene, OR) because it has a higher affinity and fluorescence upon binding than ethidium bromide. In addition, the affinity of the dye for the modified agarose is low, allowing for enhanced visualization of faint bands.

Statistical analysis. Values are means ± SD. Group means were compared by ANOVA using Fisher’s procedure post hoc analysis with StatView software for Macintosh. P < 0.05 was considered significant.

RESULTS

Hyperoxia inhibits proliferation of MLE15 cells in G2 phase of the cell cycle. MLE15 cells were chosen for study because the G1-to-S transition is disrupted by their expression of SV large-T antigen. T antigen blocks the growth inhibitory actions of p53 and Rb and alters normal associations of G1 cyclins and cyclin-dependent kinases (1, 32). Exponentially growing cultures were incubated in room air or hyperoxia, and cell number was determined every 24 h. Cell number increased in cultures exposed to room air but not in hyperoxia (Fig. 1A). The growth inhibitory effects of hyperoxia were evident within the first 24 h of exposure. Cell viability was determined by Trypan blue dye exclusion. Greater than 95% of cells exposed to normoxic conditions retained the ability to exclude dye over 72 h of culture (Fig. 1B). Although cells exposed to hyperoxia also excluded dye over the first 24 h of exposure, membrane integrity was lost rapidly with longer exposures. By 72 h, 33.3 ± 3.9% of the cells had died compared with 1.86 ± 0.38% of cells exposed to room air. Thus MLE15 cells initially ceased proliferation and then began to die when exposed to hyperoxia.

The effect of hyperoxia on cell cycle distribution was determined by flow cytometry. Asynchronous cultures exposed to room air contained cells in G1, S, and G2/M phases of the cell cycle (Fig. 2). Cells exposed to hyperoxia showed dramatic alterations in the percentage of cells in these phases. Initially, hyperoxia decreased the percentage of cells in G1 and increased the percentage in S (Table 1). By 72 h, a significant percentage of cells accumulated in G2 phase. Cells with sub-G1 content of
DNA, consistent with cell death, were not apparent in cultures exposed to room air or 24 h of hyperoxia. However, they were detected in cultures exposed to hyperoxia for 48 h with even more apparent by 72 h. The appearance of sub-G1 cells was concomitant with loss of membrane integrity by Trypan blue dye exclusion.

BrdU pulse chase labeling studies were used to determine when growth arrest occurred. Asynchronously growing cells were incubated with BrdU for 1 h to label cells in S phase (Fig. 3, top). A uniformly labeled population of cells in S phase was observed at this time. Additional cultures were washed to remove unincorporated nucleotide, and the label was chased by culturing in room air or hyperoxia for 24 h in the presence and absence of colcemid to block mitosis. The BrdU-labeled fraction of cells exposed to room air progressed through the cell cycle such that a majority of the cells had divided and returned to G1 (Fig. 3, bottom left). In contrast, labeled cells accumulated in G2 phase when cells were treated with colcemid. Similar findings were observed when the labeled cells were exposed to hyperoxia for 24 h. Labeled cells exposed to hyperoxia successfully divided and accumulated in G1 phase, whereas cells treated with colcemid arrested in G2 phase. These findings suggested that the growth inhibitory actions of oxygen occur during the second cell cycle.

This was tested by exposing BrdU-labeled cells to normoxic or hyperoxic conditions for 24 h, at which time they were incubated for an additional 24 h in the same environment in the presence or absence of colcemid. Although the fluorescent intensity decreased with each round of DNA replication, BrdU-labeled cells cultured in room air were detected in all phases of the cell cycle (Fig. 3, bottom right). This indicates that the labeled population of cells had taken on an asynchronous distribution by this time. As expected, the labeled cells cultured in room air accumulated in G2 when

Table 1. Effects of hyperoxia on cell cycle distribution

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>S</th>
<th>G2</th>
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<tbody>
<tr>
<td>Room air</td>
<td>39.4 ± 5.0</td>
<td>40.2 ± 6.4</td>
<td>20.5 ± 9.6</td>
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<tr>
<td>Hyperoxia, h</td>
<td></td>
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<tr>
<td>24</td>
<td>24.1 ± 6.4 *</td>
<td>57.6 ± 11.5 †</td>
<td>18.3 ± 7.3</td>
</tr>
<tr>
<td>48</td>
<td>9.7 ± 5.4 *</td>
<td>61.6 ± 4.3 †</td>
<td>28.7 ± 1.2</td>
</tr>
<tr>
<td>72</td>
<td>17.0 ± 1.3 *</td>
<td>49.4 ± 6.8 †</td>
<td>33.7 ± 6.9 †</td>
</tr>
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Data represent means ± SD; (n = 4 experiments). MLE15 cells cultured in room air or hyperoxia were harvested, and DNA was stained with propidium iodide. The percentages of cells in G1, S, and G2 were quantified using an Epics Profile flow cytometer. Hyperoxia significantly decreased the percentage of cells in G1 (⁎P < 0.001). It also significantly increased the percentage of cells in S and G2 phases (†P < 0.03).
treated for the second 24 h with colcemid. Cells exposed to hyperoxia for 48 h had intense fluorescent BrdU labeling present in all phases of the cell cycle. The higher fluorescent intensity of the labeled population compared with room air-exposed cells suggested that hyperoxia inhibited cell division during the 2nd day. This was confirmed by the addition of colcemid, which did not effect G2 phase arrest because the labeled cells failed to accumulate at the colcemid block. Thus growth arrest occurs during the second cell cycle.

Abrogation of the G2 checkpoint enhances oxygen-induced toxicity. Because caffeine abrogates the G2 checkpoint activated by damaged DNA (3, 13, 27, 33), its effects on oxygen-induced growth arrest were assessed. Cells were exposed to room air or hyperoxia in the presence or absence of 2 mM caffeine for 72 h (Fig. 4A). Asynchronously proliferating cultures exposed to room air or hyperoxia contained mostly cells in G1 with some in S and G2 phases. Caffeine had minimal effect on the DNA profiles of these cells, consistent with the concept that it only effects damaged cells. As shown earlier, hyperoxia decreased the percentage of cells in G2 and increased the percentage in sub-G1 and G1. Caffeine markedly reduced the number of hyperoxic cells in G2. Loss of G2 cells was associated with an increase in the percentage of cells in G1, consistent with mitosis. An increase in the sub-G1 content was also observed, indicating an increase in death of cells that failed to growth arrest. This dramatic reduction in the G2 and increase in sub-G1 and G1 populations was also observed after 24 and 48 h of hyperoxia (data not shown).

Vital dye exclusion was used to determine whether caffeine enhanced oxygen-induced toxicity. Cells were exposed to hyperoxia in the presence and absence of caffeine for 24, 48, and 72 h (Fig. 4B). As expected, cell viability was >95% in cultures exposed to room air or 24 h of hyperoxia. It rapidly decreased thereafter such that 52.4 ± 1.2% of hyperoxic cells remained viable by 72 h. Caffeine reduced viability of cells in hyperoxia at all times such that only 23.0 ± 1.1% of cells remained viable by 72 h. Caffeine did not reduce viability of cells exposed to room air (P = 0.13, data not shown).

Because caffeine abrogates the DNA damage-dependent G2 checkpoint, one would predict that it would enhance oxygen-induced DNA strand breaks. DNA laddering was used to test this hypothesis. High-molecular-weight intact DNA was obtained from cells cultured in room air, which was not noticeably affected by caffeine treatment (Fig. 5). A faint smear of lower-molecular-weight DNA was detected in cells exposed to hyperoxia for 48 h, indicating the presence of random DNA strand breaks. These strand breaks were not observed when gels were stained with ethidium bro-

**Fig. 3.** Hyperoxia exerts growth arrest in the second cell cycle. MLE15 cells were labeled for 1 h with 10 μM 5-bromo-2'-deoxyuridine (BrdU) and chased for 24 or 48 h in room air or hyperoxia. Colcemid was added to some cultures 24 h before harvesting. Bivariate distributions of BrdU incorporation vs. DNA content are shown for cells labeled for 1 h and after chasing in BrdU-free medium for 24 and 48 h. As shown in top, inset box K contains cells with fluorescent content greater than the level detected in the absence of anti-BrdU serum. Data represent findings from a single experiment repeated 2 times with similar results.
mide, which has a lower affinity for DNA than SYBR green. Caffeine increased the degree of smearing consistent with an even greater loss in DNA integrity. In fact, DNA integrity was so compromised that the smear was clearly evident by ethidium bromide staining. The appearance of smeared DNA without a distinct ladder and loss of vital dye exclusion indicates that caffeine enhanced oxygen-induced cell necrosis. Caffeine abrogates hyperoxia-induced G2 checkpoint in A549 cells. Previous studies showed that A549 cells express p53 and p21 during hyperoxia and retain more cells in G1 compared with Mv1Lu cells that fail to express p21 (23). Although this larger percentage of cells is consistent with G1 checkpoint activation, a small increase in the S and G2 population occurs during hyperoxia (Fig. 6A). Like the MLE15 cells, cell cycle profiles of A549 cells exposed to room air were not altered by caffeine. However, caffeine reduced the accumulation of G2 cells during hyperoxia and increased the presence with sub-G1 and G1 content.

Compared with MLE15 cells that die rapidly during hyperoxia, A549 cells are more resistant, as exemplified by >90% viability, even after 72 h (23). As shown in Fig. 6B, viability of cells cultured in hyperoxia for 72 h was 93.4 ± 1.5%. In contrast, caffeine enhanced oxygen-induced toxicity such that only 75.0 ± 2.2% of cells remained viable by 72 h. Caffeine did not alter viability of cultures exposed to room air (P = 0.16, data not shown).

DISCUSSION

Activation of cell cycle checkpoints in response to DNA damage is thought to provide additional time for repair to occur. Although hyperoxia damages DNA and inhibits proliferation, it is only recently appreciated that it activates the G1 checkpoint (14, 23). In this study, we provide evidence that hyperoxia also activates the G2 checkpoint. We also showed that oxygen-induced DNA strand breaks and toxicity increased when cells were exposed to caffeine, a known inhibitor of the G2 checkpoint. Because G2 is the final gatekeeper before mitosis, it is the last opportunity for cells to repair oxidized DNA before cytokinesis. Our findings emphasize the importance of checkpoint activation, during which time oxygen-induced DNA damage may be repaired.

To our knowledge, this is the first study showing that hyperoxia activates the G2 cell cycle checkpoint. Oxygen-induced G2 arrest and its sensitivity to caffeine were shown by flow cytometric analyses. The G2 delay involves inactivation and translocation of Cdc25C phosphatase to the cytosol where it can no longer activate cyclin B-dependent kinase required for mitosis. Inactivation of Cdc25C requires phosphorylation by Chk1 or Chk2, which are phosphorylated and activated by ATR and ATM, respectively (18, 33).
Trypan blue dye was determined and graphed as means ± SD of triplicate cultures. Caffeine significantly enhanced oxygen-induced toxicity at 48 and 72 h (*P < 0.002).

![Graph showing DNA histograms of cells exposed to room air or hyperoxia for 72 h in the presence and absence of 2 mM caffeine. Data are derived from a single experiment that was repeated 5 times with similar results.](image)

**Fig. 6.** Caffeine abrogates the G2 checkpoint and sensitzes A549 cells to hyperoxia. A: representative DNA histograms of cells exposed to room air or hyperoxia for 72 h in the presence and absence of 2 mM caffeine. Data are derived from a single experiment that was repeated 5 times with similar results. B: percentage of cells excluding Trypan blue dye was determined and graphed as means ± SD of triplicate cultures. Caffeine significantly enhanced oxygen-induced toxicity at 48 and 72 h (*P < 0.002).

Caffeine sensitizes cells to genotoxic stress by uncoupling the G2 checkpoint, thereby allowing damaged cells to complete mitosis (16, 28). As exemplified in baby hamster kidney cells exposed to nitrogen mustard, caffeine reduced the time allowed for repair synthesis, resulting in greater amounts of damaged DNA and reduced survival (16). DNA double strand breaks activate the G2 checkpoint. Unlike hydrogen peroxide, which causes single strand breaks, hyperoxia induces chromosome aberrations and sister chromatid exchanges (10). Double strand breaks may arise by direct oxidative attack or when a sufficient number of single strand breaks occur in close proximity. Helix instability and strand separation could occur during S phase, when replication forks form. Cells that complete S phase are predicted to have more double strand breaks compared with cells that remain in G1 or S. Although this hypothesis is unproven, MLE15 cells, which arrest in G2, are more sensitive to hyperoxia compared with A549, Mv1Lu, or HCT116 cells that arrest in G1 or S (14, 23, 24). The addition of caffeine, which allowed MLE15 cells to complete a second mitosis during hyperoxia, enhanced toxicity. Similarly, caffeine depleted the small population of A549 cells in G2 and enhanced oxygen toxicity, albeit not to the extent as in MLE15 cells. Like caffeine that blocks ATM, AT-deficient individuals and cells are extremely sensitive to IR, which involves reactive oxygen species (25). Similarly, overexpression of ATR-kinase mutant in fibroblasts enhanced sensitivity to agents that blocked DNA replication (6). These findings suggest that the G2 checkpoint protects against hyperoxia by preventing damaged DNA from undergoing mitosis before being repaired.

Most studies have focused on the G1 checkpoint, presumably because p53 and p21 increase rapidly during hyperoxia. Based on the current findings with MLE15 cells, growth arrest occurs during the second cell cycle. Hypothetically, a number of growth arrest pathways are activated during this period, including the p53-dependent G1 checkpoint. For example, A549 cells arrest in G1 and express p53 and p21 when exposed to hyperoxia (23). In contrast, Mv1Lu cells, which do not express p21 during hyperoxia, exit G1 and accumulate in S and G2. To rule out that these findings were not because of other unknown genetic differences, we investigated the effects of hyperoxia in HCT116 colon carcinoma cells and isogenic lines in which p53 or p21 was deleted by homologous recombination (14). As expected, parental HCT116 cells arrested in G1 during hyperoxia, whereas p53- or p21-deficient lines arrested in S and G2 phases. These cells were also modestly more resistant to hyperoxia, indicating that the G1 checkpoint was protective. We were, therefore, not surprised to find that MLE15 cells, which lack p53 transcriptional activity because of their expression of large-T antigen (1), failed to arrest in G1.
during hyperoxia. Similarly, SV40 transformed human microvascular endothelial cells exposed to hyperoxia arrest in G2, consistent with loss of the G1 checkpoint (unpublished observations). Interestingly, these cells are also exquisitely sensitive to hyperoxia.

Previous studies have argued that the growth inhibitory actions of hyperoxia are because of compromised energy status caused by oxygen-induced damage to mitochondria (12). Recent studies, however, revealed it inhibits proliferation through cell cycle checkpoints that are activated when DNA is damaged. The genotoxic effects of hyperoxia are clastogenic or otherwise defined as promoting chromosomal breaks (4, 11). Although these properties have been accepted for cultured cells, less is known about how hyperoxia affects DNA integrity in the whole lung. Although TUNEL staining is observed in hyperoxic mouse lungs, it is still widely interpreted as apoptosis, even though morphological signs of apoptosis, DNA laddering, and caspase activation are not detected (2). Perhaps TUNEL staining simply represents DNA strand breaks. If so, one might expect that hyperoxia inhibits proliferation in vivo through checkpoint activation. This concept is supported by our recent observation that the proliferative index of adult p21-deficient mice does not decrease during hyperoxia (21). Because the proliferative index of the adult lung is extremely low, it would be interesting to determine whether p21 exerts G1 arrest in proliferating newborn lungs. Although most experimental studies have used extremely high levels of oxygen, the effect of lower more clinically relevant levels needs further investigation. Even 40% oxygen inhibits proliferation of HeLa cells (26). Although G1 arrest is observed with extremely high levels of oxygen, bronchiole smooth muscle cells exposed to 40% oxygen accumulated in G2 (29). Thus checkpoint activation may occur at even lower levels of oxygen previously felt to be noninjurious.

In summary, we used MLE15 and A549 epithelial cells to demonstrate that hyperoxia inhibits proliferation in G1 and G2. Caffeine overrides the G2 checkpoint and sensitizes cells to oxidative damage, as shown by enhanced DNA strand breaks and cell death. Clarifying how hyperoxia activates these checkpoints and their ability to attenuate cell death may provide new avenues for curing oxidative lung injury.

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