Hyperoxia induces macrophage cell cycle arrest by adhesion-dependent induction of p21\textsuperscript{Cip1} and activation of the retinoblastoma protein.

**Summary**

Hyperoxia induces growth arrest, apoptosis, necrosis, and morphological changes (spreading and adhesion) in various types of cells. The mechanism of hyperoxia-induced cell growth arrest has not been well elucidated, especially in macrophages. One possible mechanism is a role of cell adhesion in hyperoxia-induced cell cycle arrest. To evaluate this finding, macrophages were cultured in normoxia (21% \(O_2\)) or hyperoxia (95% \(O_2\)) in adhesion or low adhesion conditions. Incubation of macrophages in hyperoxia induced cell cycle arrest. The hyperoxia-induced cell cycle arrest was prevented by low adhesion conditions. To evaluate pathways potentially involved in hyperoxia-induced growth arrest, we measured extracellular regulated kinase and retinoblastoma protein activation and p21\textsuperscript{Cip1} and p53 accumulation. Hyperoxia strongly induced activation of extracellular regulated kinase and retinoblastoma protein as well as up-regulation of p21\textsuperscript{Cip1}. These effects of hyperoxia were attenuated under low adhesion conditions, suggesting a role for integrin-dependent signaling. The induction of p21\textsuperscript{Cip1} and activation of retinoblastoma protein occurred via a p53-independent mechanism. These results suggest that adhesion-dependent pathways are required for hyperoxia-induced cell cycle arrest in macrophages.

Hyperoxia causes damage to DNA, lipids, and proteins leading to cell cycle arrest and subsequent cell death (1–7). Hyperoxia-induced growth arrest has been evaluated in epithelial cells (2–6) and endothelial cells (8, 9); however, little is known regarding the effects of hyperoxia on macrophages. What has been described is the effect of hyperoxia on stimulus-induced cytokine production (10) and hyperoxia-induced cell spreading associated with the effects on actin (13). Animal models have shown that hyperoxia induces recruitment of macrophages to the lungs (11) but decreases antibacterial function (12, 13) and cell migration (14). In this study, we evaluated the effect of hyperoxia on macrophage proliferation and cell cycle regulation.

In contrast to macrophages, more is known regarding the effects of hyperoxia on epithelial cells. In epithelial cells, hyperoxia inhibits proliferation through activation of cell cycle checkpoints until DNA damage is repaired. This phenomenon is critical for maintaining genomic integrity (3, 5, 15). Various proteins are known to be key regulators of the cell cycle, including cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors (CKI). Cyclin-CDK complexes positively regulate cell cycle progression and are negatively controlled by CKIs (16, 17). CKIs are divided into two classes on the basis of their homology, the Kip/Cip family (p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}) and the INK4 family (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p18\textsuperscript{INK4c}, and p19\textsuperscript{INK4d}). The Kip/Cip family inhibits a broad range of CDKs (17, 18). Increases in p21\textsuperscript{Cip1} (p21) have been shown to play a central role in hyperoxia-induced growth arrest (19–21). p21 has also been shown to play a crucial role in cell cycle control in response to other stimuli such as glucocorticoids (22) and cigarette smoke (23). In lung epithelial cells, a marked increase in p21 has been observed after oxygen exposure (15). A study by Corroyer et al. (15) found that one target of p21 action was the cyclin E-CDK2 complex that facilitates cell cycle progression to the S phase (15). Induction of p21 can occur via p53-dependent (24, 25) and p53-independent mechanisms (26–29). Induction of p21 independent of p53 has been reported in hyperoxia conditions (30).

Hyperoxia induces morphological changes including cell spreading (13). Previously, we reported that cell adhesion (integrin signaling) was required for lipopolysaccharide-induced activation of the mitogen-activated kinase (MAPK), extracellular regulated kinase (ERK), and optimal tumor necrosis factor-\(\alpha\) production (31). In this study, we evaluated the role of adhesion (tissue culture plates and extracellular matrix-coated plates with collagen type I, collagen type IV, or fibronectin) on hyperoxia-induced growth arrest in macrophages. The studies show that growth arrest was accompanied by increased amounts of macrophage spreading, ERK activation, p21 induction, and retinoblastoma protein (Rb) activation. These effects of hyperoxia occurred via a p53-independent mechanism and were reversed by blocking cell adhesion. These data suggest that hyperoxia-induced growth arrest requires an integrin-dependent signal.

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† To whom correspondence should be addressed: Division of Pulmonary, Critical Care, and Occupational Medicine, University of Iowa Roy J. and Lucille A. Carver College of Medicine and Veterans Administration Medical Center, Iowa City, Iowa 52242.
EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Chemicals were obtained from Sigma. Protease inhibitors were obtained from Roche Applied Science. Nitrocellulose filters were from ECL Plus, and were obtained from American Blotto, MA. Antibodies were obtained from various sources. Anti-p21 was from Santa Cruz Biotechnology (Santa Cruz, CA), and phosphorylation-specific antibodies for ERK and Rb were from Cell Signaling (Beverly, MA). Developing antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse Ig) were obtained from Santa Cruz Biotechnology. Those for scrape-resistant adherent cells (Teflon-coated plates were shipped from BioUltra Low Attachment) were obtained from Corning (Corning, NY). Extracellular matrix-coated plates with collagen type I, collagen type IV, or fibronectin were obtained from BD Biosciences (Bedford, MA).

Cell Culture and Cell Proliferation—RAW264.7 cells (TIB-71, American Type Culture Collection) were maintained at 37 °C and in Dulbecco’s modified Eagle’s medium (high glucose) with 10% fetal bovine serum and gentamicin (40 μg/ml). Cells were subcultured every 2–3 days. Experiments were performed in six-well (35 mm) Costar tissue culture plates (low-adherence or standard) or extracellular matrix-coated (collagen type I, collagen type IV, or fibronectin) plates (35 mm) under normoxia (FiO2 = 21%) or in hyperoxia (FiO2 = 95%) at the starting cell density of 0.5 × 10⁶/ml. The cells were removed by scraping, suspended in the original medium, and centrifuged at 300 × g. The cells were resuspended in 3 ml of 1× Dulbecco’s phosphate-buffered saline and counted with an electric particle counter (Coulter Electronics, Hialeah, FL).

Isolation of Whole Cell Extracts—Whole cell protein was obtained by lysing the cells on ice for 20 min in 500 μl of lysis buffer (0.05 M Tris, pH 7.5, 150 μM NaCl, 1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate; all from Roche Applied Science). The lysates were then sonicated for 20 s, incubated on ice for 30 min, and centrifuged at 15,000 × g for 10 min at 4 °C. Protein was quantitated using a protein measurement kit (protein assay kit 500-0000, Bio-Rad). Cell lysates were stored at −70 °C until use.

Western Blot Analysis—Western blot analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed on whole cell proteins. Protein (30–80 μg) was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% mercaptoethanol, 0.05% bromphenol blue, and 1.25% Tris, pH 6.8; all from Sigma), heated to 95 °C for 5 min, and fractionated on a 10 or 12.5% SDS-polyacrylamide gel run at 100 V for 90 min. Cell proteins were transferred to nitrocellulose filters by semi-dry transfer (Bio-Rad) at 20 V for 45 min. Equal loading of the protein groups on the blot was evaluated by using Ponceau S, a staining solution designed for staining proteins on nitrocellulose filters (ECL) by semi-dry transfer (Bio-Rad) at 20 V for 45 min. Equil loading of the protein groups on the blot was evaluated by using Ponceau S, a staining solution designed for staining proteins on nitrocellulose filters (ECL) by semi-dry transfer (Bio-Rad) at 20 V for 45 min. The nitrocellulose filter was blocked for 1 h in 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 h, washed, and then incubated with the primary antibody overnight. The blots were washed four times with Tris-buffered saline with Tween 20 and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody. Immunoactive bands were developed using a chemiluminescent substrate (ECL Plus or SuperSignal West Femto). An autoradiograph was obtained with exposure times of 10 s to 2 min.

Flow Cytometry—RAW264.7 cells were cultured in conditions of normoxia (21% O2) or hyperoxia (95% O2 and 5% CO2) on adhesion plates or low adhesion plates for 24 h. The cells were washed twice in phosphate-buffered saline and fixed in cold 70% ethanol overnight. After two washes in phosphate-buffered saline, the cells were treated with 0.25 mg/ml RNase A (Roche Applied Science) and stained with 50 μg/ml propidium iodide at 4 °C for 30 min in the dark and analyzed for DNA content. A total of 10,000 cells that satisfied a gate on forward and side scatter to eliminate aggregates and debris were evaluated with a FACScan flow cytometer (BD Biosciences). Data analysis was performed with ModFit LT (Verity Software House Inc, Topsham, ME).

Real-time RT-PCR—Total RNA was isolated using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. RNA was quantitated using Ribogreen Kit (Molecular Probes, Eugene, OR). 1 μg of total RNA was reverse-transcribed to cDNA using RETROscript RT-PCR kit (Ambion, Austin, TX). In a 0.2-ml PCR tube (Bio-Rad), 2 μl of cDNA (10% DNA synthesis reaction volume) was added to 48 μl of PCR reaction mixture containing 160 μM each dNTP (Invitrogen), 3.0 mM MgCl2 (Invitrogen), 1.0× SYBR Green I DNA dye (Molecular Probes), 0.2 μM of each sense and antisense primers (IDT, Coralville, IA), and 2.5 units of Platinum TaqDNA polymerase (Invitrogen). Amplification was then performed in an iCycler iQ Fluorescence Thermocycler (Bio-Rad) as follows: 5 min at 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C. Fluorescence data were captured during the 72 °C dwell time. Specificity of the amplification was confirmed using melting curve analysis. Data were collected and recorded by iCycler iQ software (Bio-Rad) and expressed as a function of threshold cycle (Ct), the cycle at which the fluorescence intensity in a given reaction tube rises above background (calculated as 10 times the mean ± S.D. of fluorescence in all of the wells over the baseline cycles). Specific primer sets used are as follows (5′ to 3′): p21 sense, TCCACGAGCATATCCAGACA; p21 antisense, CAGGGCAGGAGAAGTACCTGG; HPRT sense, CCTCATGGAACGGATTGAC; and HPRT antisense, CAGATTCAACTTGGGCGCTCA. Primers were selected based on nucleotide sequences downloaded from the National Center for Biotechnology Information data bank and designed with software by Steve Rezen and Helen J. Skatesky (1998 Primer3, code available at www-genome.wi.mit.edu/genome_software/other/primer3.html).

Quantitative Gene Expression—Quantitative p21 mRNA expression was calculated as follows. For each sample assayed, the Ct, for reactions amplifying p21 and the HPRT housekeeping gene were determined. The p21 Ct, for each sample was corrected by subtracting the Ct for HPRT (ΔC). Finally, p21 mRNA abundance, relative to HPRT mRNA abundance, was calculated by the formula 2−ΔCt. Validity of this approach was confirmed by using serial 10-fold dilutions of template for p21 and HPRT. Using the 10-fold dilutions, the amplification efficiencies for p21 and HPRT were found to be identical.

Statistical Analysis—The results were expressed as the mean ± s.E. The number of cells in each condition at various time points in adher- ence or non-adherence conditions was compared using an unpaired Student’s t test.

RESULTS

Hyperoxia Induces Macrophage Growth Arrest—To investigate the effects of hyperoxia on RAW264.7 cells (macrophages), we performed the following experiments. Macrophages were cultured in standard tissue culture plates and extracellular matrix-coated (collagen type I, collagen type IV, or fibronectin) plates under conditions of normoxia (21% O2) or hyperoxia (95% O2) at the starting cell density of 0.5 × 10⁶/ml. Cell counts were measured at various time points as described under “Experimental Procedures.” As shown in Fig. 1A, hyperoxia-induced cell growth arrest was significant at 24 and 48 h (p < 0.05) in both standard tissue culture plates and extracellular matrix-coated plates with collagen type I, collagen type IV, or fibronectin. Fig. 1B shows morphological changes demonstrating a greater degree of cell spreading in the hyperoxia group on all of the substrates at 48 h. These studies show that hyperoxia decreases cell proliferation and induces morphological changes in macrophages.

Hyperoxia-induced Growth Arrest Requires Cell Adhesion—Because hyperoxia induced marked morphological changes associated with cell spreading (13), we postulated that an increase in cell adhesion by hyperoxia might contribute to cell growth arrest. To evaluate this possibility, macrophages were cultured in normoxia (21% O2) or hyperoxia (95% O2) in standard tissue culture plates or low adherence plates. The number of cells was measured at 2, 24, and 48 h. As shown in Fig. 2A, low adhesion conditions blocked hyperoxia-induced cell growth arrest. These results suggest that hyperoxia-induced cell growth arrest is dependent on cell adhesion.

Hyperoxia Induces Growth Arrest at the G1/S Phase of the Cell Cycle—Hyperoxia has been reported to induce cell cycle arrest and apoptosis or necrosis (1–7, 32). To determine whether hyperoxia induced adhesion-dependent cell cycle arrest, we performed flow cytometry with DNA staining by propidium iodide. Cell cycle was analyzed at 24 h with four conditions, adhesion conditions with normoxia (21% O2) or hyperoxia (95% O2) and low adhesion conditions with normoxia or hyperoxia. Cell cycle analysis showed that hyperoxia induced a significant decrease in the S phase at 24 h (Fig. 2B) associated with cell cycle arrest at the G1/S phase (data not shown). There was no evidence for apoptosis in hyperoxia con-
ditions up to 48 h (data not shown). Taken together, the data show that hyperoxia induces an adherence-dependent G1 phase cell growth arrest with limited cell death.

Hyperoxia Induces ERK Activity That Is Dependent on Adhesion—Hyperoxia has been shown to induce the activation of ERK MAPK (32). Previously, we showed that cell adhesion was necessary to induce optimal activation of ERK in macrophages stimulated by lipopolysaccharide (31). Based on these observations, we hypothesized that hyperoxia-induced activation of ERK might be attenuated by low adhesion. To evaluate this hypothesis, macrophages were cultured in adhesion or low adhesion conditions with normoxia (21% O2) or hyperoxia (95% O2 and 5% CO2) at the starting cell density 0.5 × 10^6/ml. The cell counts were measured at various time points (2, 24, and 48 h). Data are expressed as mean ± S.E. for three independent experiments (*, p < 0.05). B, RAW264.7 cells were treated as in A, and the digital photographs were obtained at 48 h. The photographs represent one of three identical experiments.

![Figure 1](http://www.jbc.org/)

**Fig. 1. Hyperoxia induces macrophage growth arrest.** A, RAW264.7 cells were cultured on regular tissue culture plates or extracellular matrix-coated (collagen type I, collagen type IV, or fibronectin) under normoxia (21% O2) or hyperoxia (95% O2 and 5% CO2) at the starting cell density 0.5 × 10^6/ml. The cell counts were measured at various time points (2, 24, and 48 h). Data are expressed as mean ± S.E. for three independent experiments (*, p < 0.05). B, RAW264.7 cells were treated as in A, and the digital photographs were obtained at 48 h. The photographs represent one of three identical experiments.

Western blot analysis was performed for phosphorylation of ERK. Hyperoxia induced a sustained markedly increased phosphorylation of ERK in cells cultured in adhesion conditions (Fig. 3A). Notably, low adhesion reversed the hyperoxia-induced ERK activity as shown in Fig. 3B. Pharmacological inhibition of ERK led to hyperoxia-induced cell death (data not shown), not increased cell proliferation. This finding suggests that the cell cycle arrest is not the result of the hyperoxia-induced ERK activity and that other hyperoxia-inducible factors may play a role in cell cycle arrest. As a composite, these data suggest that hyperoxia-induced ERK activation is adhesion-dependent and may play a role in cell survival in hyperoxia.
Hyperoxia and Cell Adhesion

A. Cell Number

![Cell Number Graph]

B. Cell Cycle

![Cell Cycle Graph]

**Fig. 2.** Hyperoxia-induced growth arrest requires cell adhesion. A, RAW264.7 cells were cultured in conditions of normoxia (21% O2) or hyperoxia (95% O2 and 5% CO2) on adhesion plates to measure cell counts. Data are expressed as mean ± S.E. for three independent experiments (*, p < 0.05, normoxia/adhesion compared with hyperoxia/adhesion). B, RAW264.7 cells were treated as in A, and flow cytometry with DNA assay was performed to determine the phosphorylation state of Rb. As shown in Fig. 4, hyperoxia decreased phosphorylation of Rb. The decrease in hypophosphorylated Rb did not occur in low adhesion conditions. These results are consistent with the p21 data and cell cycle analyses shown above. As a composite, the data suggest that hyperoxia requires adhesion-dependent signals to induce p21, activate Rb, and cause cell cycle arrest.

**DISCUSSION**

This study demonstrates that in macrophages, hyperoxia inhibits cell proliferation and induces morphological changes such as cell spreading. Hyperoxia-induced growth arrest occurred in both standard tissue culture plates and extracellular matrix-coated plates with collagen type I, collagen type IV, or fibronectin. The hyperoxia-induced growth arrest was accompanied by p21 induction and Rb activation. All of these observations were dependent on cell adhesion as cells cultured in low adhesion conditions continued to proliferate and did not increase p21 or Rb activity. The effects were not dependent on p53 activity. Interestingly, hyperoxia induced ERK activity and this was blocked by low adhesion conditions. Inhibition of ERK activity, however, did not reverse the growth arrest but facilitated cell death, suggesting that hyperoxia-induced ERK was important for cell survival under hyperoxia conditions. As a composite, these data show that in macrophages, hyperoxia/adhesion induces cell survival in part via an effect on ERK MAPK and cell cycle arrest via an effect of p21 induction and Rb activation.

A number of studies have evaluated the effects of hyperoxia on cell cycle in non-macrophage cell lines. It has been reported that hyperoxia inhibits cell proliferation and induces cell death in A549 epithelial cells (2, 6), MLE15 murine type II epithelial cells (3), HCT116 colon carcinoma cells (4), Mv1Lu pulmonary adenocarcinoma cells (5, 6) and HeLa cells (7). However, few studies have examined the effect of hyperoxia on cell cycle and survival in macrophages. Human alveolar macrophages proliferate at very low levels in normal lungs. A study of 14 normal subjects showed no significant change in the number of alveolar macrophages in response to hyperoxia (>95% O2) for 17 h (35). In contrast, Nerurkar et al. (36) investigated the effect of hyperoxia on the cell cycle in rabbit alveolar macrophages. Alveolar macrophages exposed to hyperoxia for 18 h increased incorporation of [3H]thymidine and proliferation (36). Thus, the effects of hyperoxia on macrophages in terms of cell cycle and survival remain poorly defined. RAW264.7 cells were chosen for this study because they are a well developed macrophage cell line that proliferates rapidly and has been used as a model for cell cycle analyses. We found that hyperoxia induces G1 phase cell cycle arrest and marked morphological changes such as cell spreading in macrophages. These changes were dependent on signals downstream of cell adhesion. Our data suggest a novel mechanism of cell growth control, i.e., adhesion-dependent hyperoxia-induced cell cycle arrest. The growth arrest did not result in cell death during the time frame studied, potentially because of the hyperoxia-induced ERK activation.

Previously, we found that ERK (p42/p44) activity was regulated by cell adhesion in macrophages (31). Moreover, it has been reported that ERK activity can be induced by hypoxic exposure and leads to hyperoxia-induced apoptosis (32). Our studies showed that hyperoxia-induced ERK activity was me-
mediated by cell adhesion and appeared to maintain cell viability. We observed minimal cell death under conditions of hyperoxia unless ERK activity was inhibited. Therefore, we speculate that activation of ERK by hyperoxia in macrophages functions as a survival signal rather than a pro-apoptotic signal as suggested by other studies (37–39).

A report by Corroyer et al. (15) describes a marked increase in p21 in lung epithelial cells of rats under hyperoxia. p21 has been shown to play a dual role in cell cycle progression. It acts as an inhibitor of the cyclin E-CDK2 complex, which facilitates cell cycle progression to S phase (15), and as an assembly activator of cyclin D-CDK4 complexes (40, 41). Activation of p21 also induces cell cycle arrest by preventing DNA synthesis. p21 binds to proliferating cell nuclear antigen, a co-activator of DNA polymerases δ and ε (42), and modulates the primer template recognition complex, which inhibits DNA replication (43). p21 has been suggested to play a role not only in the functions of cell cycle control but also in enhancing cell survival and differentiation. Several studies have shown increased susceptibility to p53-mediated apoptosis in p21-deficient cells (44, 45). Moreover, ectopic p21 expression in p21-deficient mouse embryonal fibroblasts protected against p53-induced apoptosis (45). Asada et al. (46) have reported that ectopic expression of p21 in U937 cells resulted in monocytic differentiation and resistance to various apoptotic stimuli (46).

p21 can be induced by p53-dependent (24, 25) and/or p53-independent mechanisms (26–29). We found that hyperoxia induced p21 accumulation by a p53-independent mechanism.
Hyperoxia induces p21 accumulation. A, RAW264.7 cells were cultured in conditions of normoxia (21% O₂) or hyperoxia (95% O₂ and 5% CO₂) on adhesion plates or low adhesion plates for 48 h. Western blot analysis was performed for p21 in the whole cell lysates. B, RAW264.7 cells were treated as in A, and total RNA was isolated at 24 h. p21 mRNA quantitative RT-PCR was performed as described under “Experimental Procedures.” Data are expressed as mean ± S.E. for four independent experiments (**, p < 0.01, normoxia/adhesion compared with hyperoxia/adhesion). C, RAW264.7 cells were treated as in A, and Western blot analysis was performed for p53 in the whole cell lysates. Densitometries of p21 and p53 are shown as fold increase (mean optical density units in experimental sample/mean optical density units in normoxia/adhesion sample). Western blotting data are representative of three experiments.
Although p53 has been suggested to play a critical role in p21 induction following DNA damage (47), several studies have shown p53-dependent pathways mediated by various transcription factors including Sp1 (26), activator protein 2 (27), STAT1 (28), and CCAAT/enhancer-binding protein (29). O’Reilly et al. (30) have shown that in vivo exposure of lungs to hyperoxia increases p53-independent expression of p21 in p53-deficient mice but to a lesser extent than observed for p53-independent induction of p21 protein, activation of Rb, and cell cycle arrest in murine RAW264.7 cells were cultured in conditions of normoxia (21% O2) or hyperoxia (95% O2 and 5% CO2) on adhesion plates or low adhesion plates for 24 h. Western blot analysis was performed for hyperphosphorylation of Rb (inactive state). Densitometry of hyperphosphorylated Rb is shown as fold increase (mean optical density units in experimental sample/mean optical density units in try of hyperphosphorylated Rb (inactive state). Densitometry data are representative of three experiments.

In conclusion, this study demonstrates that hyperoxia induces cell cycle arrest that is dependent on cell adhesion. Hyperoxia induces both p21 accumulation and Rb activation, also in an adhesion-dependent manner. Hyperoxia also induces ERK activation via an adhesion-dependent mechanism that was linked to cell survival under hyperoxia conditions. The novel observations of this study, adhesion-dependent induction of p21 protein, activation of Rb, and cell cycle arrest suggests a previously unknown role for integrin signaling in macrophage responses to hyperoxia.

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Toru Nyunoya, Linda S. Powers, Timur O. Yarovinsky, Noah S. Butler, Martha M. Monick and Gary W. Hunninghake

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