Oxygen regulation of arterial smooth muscle cell proliferation and survival

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1Institute of Medical Science, 2Toronto General Hospital Research Institute and The Heart and Stroke/Richard Lewar Centre of Excellence, 3Department of Medical Biophysics, University of Toronto; 4Division of Respirology, Department of Medicine, St. Michael’s Hospital and the University of Toronto; 5Terrence Donnelly Vascular Biology Laboratories, 6Keenan Research Centre of the Li Ka Shing Knowledge Institute of St. Michael’s Hospital, Toronto, Canada

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Basu Ray J, Arab S, Deng Y, Liu P, Penn L, Courtman DW, Ward ME. Oxygen regulation of arterial smooth muscle cell proliferation and survival. Am J Physiol Heart Circ Physiol 294: H839–H852, 2008. First published November 30, 2007; doi:10.1152/ajpheart.00587.2007.—The purpose of this study was to determine if hypoxia elicits different proliferative and apoptotic responses in systemic arterial smooth muscle cells incubated under conditions that do or do not result in cellular ATP depletion and whether these effects are relevant to vascular remodeling in vivo. Gene expression profiling was used to identify potential regulatory pathways. In human aortic smooth muscle cells (HASMCs) incubated at 3% O2, proliferation and progression through the G1/S interphase are enhanced. Incubation at 1% O2 reduced proliferation, delayed G1/S transition, increased apoptotic cell death, and is associated with mitochondrial membrane depolarization and reduced cellular ATP levels. In aorta and mesenteric artery from rats exposed to hypoxia (10% O2, 48 h), both proliferation and apoptosis are increased, as are medial nuclear density and smooth muscle cell content. Although nuclear levels of hypoxia-inducible factor 1-α (HIF-1α) are increased to a similar extent in HASMCs incubated at 1 and 3% O2, expression of tumor protein p53, its transcriptional target p21, as well as their regulatory factors and downstream effectors, are differentially affected under these two conditions, suggesting that the bidirectional effects of hypoxia are mediated by this pathway. We conclude that hypoxia induces a state of enhanced cell turnover through increased rates of both smooth muscle cell proliferation and death. This confers the ability to remodel the vasculature in response to changing tissue metabolic needs while avoiding the accumulation of mutations that may lead to malignant transformation or the formation of abnormal vascular structures.

ARTERIAL SMOOTH MUSCLE CELLS are exposed to a broad range of oxygen concentrations. In the aortic wall, O2 concentrations from 11.2% at the lumen to 2.2% at a depth of 150 μm (5, 56) have been recorded, and longitudinal gradients of similar magnitude occur in the normal microcirculation (69). Regions of severe hypoxia (≤1% O2) exist subjacent to atherosclerotic plaques (6) and in arteries of hypertensive (15) and diabetic (55) rodents. Smooth muscle cell proliferation and survival are modulated by hypoxia (10, 13), leading to speculation about their role in vasculogenesis (38), vascular remodeling (54), and atherosclerosis (10, 13, 60, 61). The effects of hypoxic incubation on vascular smooth muscle cells in culture, however, have been inconsistent with both enhanced proliferation (13, 25, 59) and growth arrest and apoptosis (19, 21, 23, 57) being reported. More importantly, the effect of levels of hypoxemia relevant to human cardiopulmonary disease on systemic arterial smooth muscle cell turnover in vivo is unknown.

Inconsistencies notwithstanding, the experimental evidence supports an important role for oxygen in regulating vascular smooth muscle cell growth and survival, which, given its physiological and clinical relevance, requires clarification. Since cell replication is highly energy dependent (74), it is intuitive, though unproven, that hypoxia may have different effects depending on the degree to which cellular energy status is compromised. Furthermore, although a number of cell cycle regulatory genes are oxygen sensitive, discrepant results have prevented the development of a unifying hypothesis that accounts for the divergent effects observed. This study was carried out to determine whether different responses are elicited in human aortic smooth muscle cells (HASMCs) subjected to hypoxic incubation under conditions, which do or do not result in cellular ATP depletion, and whether these effects are relevant to vascular remodeling during hypoxia in vivo and to identify potential regulatory pathways by using gene expression profiling in cells exposed to conditions that elicit discordant responses.

MATERIALS AND METHODS

Antibodies and Reagents

Fluorescein isothiocyanate (FITC)-conjugated Ki67 antibody was obtained from Dako (Glostrup, Denmark), hypoxia inducible factor 1-α (HIF-1α) antibody from Novus Biologicals (Littleton, CO), and cell division cycle 6 (CDC6) antibody from Lab Vision (Fremont, CA). Mini chromosome maintenance 2 (MCM2) and p21 antibodies were from BD Pharmingen (San Diego, CA). P53 antibody was from Cell Signaling Technology (Danvers, MA) and telomerase reverse transcriptase (TERT) antibody from Calbiochem (San Diego, CA). CaspASE FITC-VAD-fmk in situ marker and TUNEL kits were both from Promega (Madison, WI), 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) labeling kit, ATP bioluminescence assay kit, and TO-PRO-3 dye were purchased from Molecular Probes (Carlsbad, CA). All other reagents were from Sigma (St. Louis, MO).

Cell Culture Studies

HASMCs and A7R5 rat aortic smooth muscle cells (Cambrex Bio Science, Walkersville, MD) were propagated to passage 6 in an atmosphere of 21% O2, 5% CO2, and balance N2 at 37°C. HASMCs were grown in smooth muscle growth medium (SMGM-2) (Cambrex) consisting of smooth muscle basal medium (SMBM) supplemented...
with single quots of 0.1% insulin, 0.2% human FGF-B, 0.1% GA-1000 (gentamicin and amphotericin B), 5% vol/vol FBS, and 0.1% human EGF. A7r5 cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (200 U/ml), and streptomycin (0.2 mg/ml). All cells were grown to 70% confluence under normoxic conditions (air-5% CO2, culture media O2 concentration = 20.5%). The media was then changed, and cells were placed in a humidified Plexiglas chamber (Billups Rothberg, San Diego, CA) maintained at 37°C and continuously flushed with gas mixtures containing air-5% CO2 or 10, 5, 3, or 1% O2-5% CO2-balance N2. Experiments were repeated three times with six replicates per observation. HASMCs were from at least three human donors.

Differences in timing, duration, and severity of hypoxic exposure may explain, in part, the conflicting results obtained in previous studies; however, the precise elements of the experimental paradigm that contribute to this variability are unknown. In the present study, cells were exposed to hypoxia according to two protocols so that proliferation and cell death could be each assessed by different independent assays, using both methods. Concordance between the findings, regardless of the protocol chosen, provides assurance that the observed effects are not due to differences in the method of hypoxic exposure. In some studies, the total duration of incubation was constant, whereas the duration of hypoxic exposure before harvesting was varied. Cells were grown to 70% confluence under normoxic conditions and then exposed to the following: 1) a further 48 h of normoxia, the control condition, 2) 32 h of normoxia followed by 16 h of hypoxia, the 16-h hypoxic condition, or 3) 48 h of hypoxia, the 48-h hypoxic condition. Cells were harvested on the same day and analyzed simultaneously, thus minimizing intraexperimental error. Cell counting studies, cell cycle analysis, measurement of proliferation markers (Ki67, CDC6, and MCM2), annexin V-propidium iodide (PI) staining, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL), and microarray analyses were performed on cells treated in this manner. In the second protocol, cells incubated to 70% confluence under normoxic conditions were then incubated for 16 or 48 h under either normoxia or hypoxia. Results obtained in cells, incubated at each O2 concentration and duration, were compared with their own corresponding normoxic control values. This method was used to assess the effects of hypoxia on [3H]thymidine incorporation, mitochondrial membrane depolarization, intracellular ATP concentration, and nuclear HIF-1α, p53, and p21 protein levels.

Cell counting. The net effect of each condition on total cell number was assessed by cell counting. After exposure to normoxia or hypoxia (10, 5, 3, or 1% O2) for 16 or 48 h, cells were washed twice with HBSS, detached with 0.25% trypsin and 0.02% EDTA, and counted using a standard hemacytometer (American Optical, Buffalo, NY). Cell viability was assessed by Trypan Blue exclusion. Initial studies indicate that the effects of 3 and 1% O2 differ qualitatively (Fig. 1) a further 48 h of hypoxia, the 16-h hypoxic condition, or 3) 48 h of hypoxia, the 48-h hypoxic condition. Cells were harvested on the same day and analyzed simultaneously, thus minimizing intraexperimental error. Cell counting studies, cell cycle analysis, measurement of proliferation markers (Ki67, CDC6, and MCM2), annexin V-propidium iodide (PI) staining, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL), and microarray analyses were performed on cells treated in this manner. In the second protocol, cells incubated to 70% confluence under normoxic conditions were then incubated for 16 or 48 h under either normoxia or hypoxia. Results obtained in cells, incubated at each O2 concentration and duration, were compared with their own corresponding normoxic control values. This method was used to assess the effects of hypoxia on [3H]thymidine incorporation, mitochondrial membrane depolarization, intracellular ATP concentration, and nuclear HIF-1α, p53, and p21 protein levels.

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To determine whether the effect of hypoxia persists during extrinsic growth factor stimulation, as would pertain in vivo (43, 44), the effect of hypoxia during stimulation with platelet-derived growth factor (PDGF) was assessed. HASMCs were incubated under normoxia for 48 h following which, the cell culture medium was replaced with medium containing 0.5% FBS. Forty-eight hours later, 10 nM PDGF-BB (R&D Systems, Minneapolis, MN) or the same volume of diluent (100 μM SMGM-0.5% FBS) was added to the medium, and the cells were allowed to proliferate under either normoxic or hypoxic (1 or 3% O2) conditions for 48 h.

Fig. 1. Effects of hypoxia on human aortic smooth muscle cell (HASMC) numbers after incubation for 16 and 48 h (A) and the proliferative response to 10 nM platelet-derived growth factor (PDGF)-BB (B and C); n = 6 per condition. *P < 0.05 vs. corresponding normoxic or hypoxic values in the absence of PDGF.
added to each well, and the cells were exposed to air, 1 O₂ or 3 O₂ for 16, 24, 48, or 72 h. Following each exposure, the cells were washed thrice with phosphate-buffered saline (PBS) and fixed with ice-cold 10% (wt/vol) trichloroacetic acid for 20 min. The resulting precipitate was solubilized in 0.1 N NaOH (0.5 ml/well) at 37°C. Solubilized DNA was transferred into scintillation vials, and [3H]thymidine incorporation was quantified by scintillation counting (Liquid Scintillation System, Beckman Instruments). This experiment was repeated three times using cells from three human donors with six replicates per observation.

**Ki67 protein.** The presence of Ki67, a nuclear protein expressed only in proliferating cells, was detected in cells grown under normoxic or hypoxic (1 or 3% O₂) conditions for 16 or 48 h as an additional marker of the effect of hypoxia on HASMC proliferation. Detached cells were centrifugated at 1,500 rpm for 10 min at 20°C. The supernatant was removed, and the pellet was suspended in 200 μl of membrane-shredding solution (99.95% vol/vol Ca²⁺- and Mg²⁺-free Dulbecco’s PBS, 0.5% vol/vol NP-40, 0.5 mM Na₂-EDTA, 2H₂O, 0.5% wt/vol BSA, 20 μg/ml protease inhibitor, and 0.2 mg/ml RNAase and kept at room temperature for 15 min. Samples were incubated with 10 μl of antibody in the dark for 30 min and analyzed by flow cytometry (Model Epics Altra; Beckman Coulter, Fullerton, CA). A minimum of 10,000 events per sample was recorded, and cell debris was excluded by adjusting the forward light scatter threshold setting. The number of cells positive for Ki67 was calculated using CELLQuest software (Becton Dickinson, St. Louis, MO).

**Annexin V-PI labeling.** Changes in cell number may reflect effects on cell death (apoptosis and/or necrosis) as well as proliferation. To assess the effect of hypoxic incubation on apoptosis, the Roche (Basel, Switzerland) annexin V-fluorescence (Fluos) staining kit was used to detect phosphatidylserine externalization (an early event in apoptosis), and PI uptake was used as a marker of cell death in HASMC exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h. Cell suspension was centrifuged at 1,500 rpm for 10 min at 4°C. The pellet was resuspended in 5 ml of cold PBS and centrifuged again. The cell suspension was centrifuged at 1,500 rpm for 10 min at 4°C. The supernatant was removed, and the pellet was resuspended in annexin V-Fluos labeling solution (20 μl annexin V-Fluos labeling reagent and 20 μl PI solution per milliliter of incubation buffer) at 37°C. Labeled cells were analyzed by flow cytometry, and the percentage of cells positive for either annexin V, PI, or both were calculated.

**Caspase activation.** Caspase activation is the final common step in apoptosis and therefore identifies cells in which this process has been initiated. Caspase activation was detected in HASMCs exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h using CaspACE FITC-VAD-fmk, a FITC conjugate of the cell-permeable inhibitor of caspases. This structure allows delivery of the inhibitor into the cell, where binding to activated caspase serves as an in situ marker for apoptosis. About 2×10⁶ cells were incubated with 100 μM FITC-VAD-fmk at room temperature in the dark for 20 min. Cells were then washed, resuspended in PBS, and the percentage of cells positive for activated caspase quantified by flow cytometry.

**TUNEL.** DNA fragmentation is characteristic of cells dying of apoptosis and was detected in HASMCs using the DeadEnd Fluorometric TUNEL System, Roche. Cells exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h were centrifugated at 1,500 rpm for 10 min at 4°C. The supernatant was removed and the cells fixed in 1% wt/vol iced paraformaldehyde for an hour. The cells were washed with PBS and 70% ethanol. DNA-labeling solution [terminal deoxynucleotidyl transferase (TdT) enzyme and FITC-DUTP] was added and samples kept at 37°C for an hour, after which they were analyzed for DNA breaks by flow cytometry.

**DNA content and/or cell cycle analysis.** The changes in DNA content associated with progression through the cell cycle were quantified by PI staining to determine the effect of hypoxic incubation on the fraction of the cell population in each cell cycle phase (G0/G1, S, and G2/M). HASMCs exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h were trypsinized, pelleted, resuspended, and washed once in cold PBS. The cells were fixed with 70% ethanol and maintained at 4°C for 60 min. Ethanol was washed out, and cells were resuspended in 1 ml of PBS and 10 μl RNAse and incubated for 45 min at room temperature. PI (10 μl) was added, and cells were incubated in the dark at room temperature for 30 min before analysis by flow cytometry. In each experiment, 10,000 cells were counted. The amplitude of the fluorescent signal was analyzed to quantify DNA content.

**Mitochondrial membrane potential.** Depolarization of the mitochondrial membrane, resulting from and contributing to failure of aerobic ATP synthesis, is an early event in apoptosis that precedes mitochondrial release of caspase-activating factors and was detected by a cytofluorometric method by using the potential-sensitive probe JC-1. The JC-1 monomer enters the mitochondria at physiological membrane potentials (62), where, as a result of aggregation, its emission wavelength changes from 530 nm (green) to 590 nm (orange) when excited at 490 nm. Disaggregation to the monomeric form during mitochondrial membrane depolarization is detected as an increase in green emission. HASMCs exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h were incubated with JC-1 (10 μg/ml) for 20 min in the dark, washed, and resuspended in 1 ml PBS. The percentage of cells positive for JC-1 monomers was quantified by flow cytometry.

**Intracellular ATP concentration.** ATP concentrations were measured as an index of cellular energy status. HASMCs exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h were washed twice with ice-cold PBS and lysed by the addition of equal volumes of 3.6% perchloric acid. Samples were centrifuged, and ATP concentrations in the supernatants were determined by using an ATP bioluminescence assay (Molecular Probes), according to the instructions provided by the manufacturer. The photometer was set for a 5-s delay period and a 5-s integration period. ATP levels were calculated by using standard reference solutions corrected for background luminescence.

**Western blot analysis.** Western blot analysis was used to quantify levels of HIF-1α, the markers of CDC6 (35) and MCM2 (36), which form the prereplication complex at the initiation site for DNA synthesis, and cell-cycle regulatory proteins p21, p53, and telomerase subunit TERT in nuclear extracts from HASMCs exposed to normoxia or hypoxia (3 or 1% O₂) for 16 or 48 h. Cells were lysed in buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1% Nonidet P-40 (NP-40)] with protease inhibitors (5 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 0.5 mM Pefabloc, and 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, and 20 mM glycero phosphate). Nuclear proteins were then extracted with buffer B [50 mM HEPES (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5 mM MgCl₂, and 20% glycerol], containing both protease and phosphatase inhibitors. Equal amounts of protein extracted from HASMCs, incubated under normoxic and hypoxic (3 or 1% O₂) conditions for 16 and 48 h, were loaded on 4–12% Tris-glycine gels, separated by electrophoresis, and proteins were then extracted with buffer B [50 mM HEPES (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5 mM MgCl₂, and 20% glycerol], containing both protease and phosphatase inhibitors. Equal amounts of protein extracted from HASMCs, incubated under normoxia and hypoxia (3 or 1% O₂) for 16 or 48 h, were loaded on 4–12% Tris-glycine gels, separated by electrophoresis and transferred to nitrocellulose. Membranes were blocked with 5% milk overnight and probed with anti-CDC6 (1:2,000), anti-MCM2 (1:2,000), anti-HIF-1α (1:500), anti-p21 (1:3,000), anti-p53 (1:1,000) and anti-TERT (1:200). In all cases, protein concentration was determined by the Bradford assay, and appropriate volumes of extraction buffer to produce constant protein loading in each lane were mixed with SDS loading buffer. Equality of protein loading and transfer efficiency were corroborated by full-lane densitometry of the Ponceau red-stained membranes. Immunoblots were probed with horseradish peroxidase-donkey anti-rabbit IgG (1:1,000 in blocking buffer) and visualized by enhanced chemiluminescence (ECL Plus kit, Amersham Biosciences). Band intensity was quantified by densitometry (Bio-Rad Laboratories, Mississauga, ON, Canada).

**Microarray analysis using Affymetrix GeneChip hybridization.** Gene expression profiling was used to identify potential regulatory pathways in cells exposed to 3 and 1% O₂, conditions that elicit
discordant proliferative and apoptotic responses. In three separate experiments total RNA was isolated from HASMCs exposed to normoxia, 3% O2 and 1% O2 for 16 and 48 h using Trizol Reagent (GIBCO-BRL). The quality of RNA was assessed with the use of an Agilent 2100 Bioanalyzer (version A.02.01S1232, Agilent Technologies). Hybridizations were performed on the HG-U133A GeneChip Set with a total of 22,280 genes (Affymetrix, Santa Clara, CA). Samples were prepared for hybridization (six hybridizations per experiment), according to standard Affymetrix instructions, and performed at the Toronto Genomic Core Centre at the Hospital for Sick Children. Experimental design, gene lists, hierarchical trees, chip hybridizations, and statistical analyses were in compliance with the Minimum Information About a Microarray Experiment (MIAME) guidelines (7). Data obtained from the GeneChip operating software analyses of the individual arrays were normalized using the robust multichip analysis method. After filtering, two-way ANOVA (non-equal variance) was performed, and differentially regulated genes were clustered by using GeneSpring 7.0. For details see the supplementary data (GEO accession no. GSE 4725).

Animal Studies

The effects of hypoxic exposure on smooth muscle cell proliferation and apoptosis in the systemic circulation in vivo were assessed in male Sprague-Dawley rats (175–200 g). All protocols were in compliance with standards set by the Canadian Council of Animal Care and were approved by the Institutional Animal Care Committee. Rats were placed in a Plexiglas chamber into which the flow of air and nitrogen was controlled independently. Rats exposed to hypoxia breathed a gas mixture containing 10% O2 for 48 h. Normoxic control animals breathed room air under otherwise identical conditions. In preliminary experiments, the arterial PO2 in rats breathing 10% O2 averaged 38 Torr (range, 35–42 Torr) (3). Data from each animal were averaged to serve as a single value for statistical analysis.

Detection of nonviable aortic and mesenteric artery smooth muscle cells. Nonviable cells were detected by their failure to exclude PI. As a positive control lipopolysaccharide (LPS) from Escherichia coli 055:B5 (0.1 mg/kg body wt) was injected into the right jugular vein 72 h before euthanasia, to induce apoptosis. At the end of the exposure period, normoxic, LPS-treated, and hypoxia-exposed rats were anesthetized by intramuscular injection of 10%30 mg/ml ketamine (100 mg/ml), followed by intravenous injections of 5 µmol/kg PI. After 15 min, an incision was made at the left atrial appendage of the heart and flushed thoroughly with 300 ml PBS. En face sections of aorta and mesenteric artery were perfusion fixed with 3.7% paraformaldehyde for 1 h, washed with PBS and, permeabilized with 0.2% Triton X-100. TO-PRO-3 was used for nuclear counterstaining. Sections were then mounted on glass slides with glycerol-PBS and viewed under a laser-scanning confocal microscope (Bio-Rad). Cells that did not exclude PI, as indicated by nuclear fluorescence, were considered nonviable.

Detection of apoptotic cells. DNA fragmentation in aortic and mesenteric artery sections was detected by TUNEL (DeadEnd Fluorometric TUNEL System, Roche). Slides containing paraffin-embedded sections (normoxic and hypoxic rat aorta and mesenteric artery) were de-waxed, rehydrated, permeabilized with proteinase K, preincubated with equilibration buffer, and incubated with labeling solution (rTdT and nucleotide mixed with fluorescein-labeled dUTP) for 1 h at 37°C. The reaction was terminated by incubating samples in a stopping buffer for 30 min. After PBS washes and counterstaining with TO-PRO-3, the samples were mounted and examined by laser confocal microscopy.

Detection of proliferating cells. To determine whether hypoxia induces aortic and mesenteric artery smooth muscle cells to proliferate in vivo, bromodeoxyuridine (BrdU) uptake by these cells was assessed as a marker of de novo DNA synthesis. BrdU was infused subcutaneously using osmotic pumps (model no. 2ML-2; Alza Corp, Palo Alto, CA). Pumps containing 0.32 g of BrdU in 2 ml vehicle (0.4% DMSO) were implanted intrascapularly in normoxic and hypoxia-exposed rats 48 h before euthanasia. The rats received ~0.4 mg BrdU/h delivered continuously. At the end of the labeling period, thoracic aorta and mesenteric artery segments were excised, fixed with paraformaldehyde, washed with PBS, dehydrated in graded ethanol (70–100%), cleared in xylene, and embedded in paraffin. Sections (5-µm thick) were cut on an oscillating blade microtome (Leica, Wetzlar, Germany) and placed on coated glass microscope slides (Fisher Scientific, Pittsburgh, PA). Dewaxed and rehydrated slides were incubated with 2 M HCl for 1 h at 37°C. The acid was neutralized by 0.1 M borate buffer, pH 8.5. Following PBS washes, the slides were incubated with fluorescein-labeled anti-BrdU antibody (Roche) for 1 h at room temperature, protected from light. Slides were then washed with PBS and mounted for microscopy.

Quantitative analysis. The number of TUNEL-positive, BrdU- and TO-PRO-3-labeled cells was determined in three aortic and mesenteric artery sections from each of six animals from each experimental group. Four to six randomly selected microscopic fields ranging from 90–300 cells per field were counted for each section, and the number of TUNEL- or BrdU-positive nuclei were expressed as a percentage of the total number of nuclei. Nuclear density was determined using Image J analysis software and expressed as the number of nuclei per mm² × 10⁻⁴. Data from each animal in each group were averaged to serve as a single value for statistical analysis.

Smooth muscle α-actin staining. The net effect of in vivo hypoxic exposure on smooth muscle cell content in aorta and mesenteric artery was assessed by immunohistochemical staining for smooth muscle α-actin. Aortic and mesenteric artery sections from normoxic and hypoxic rats were embedded in optimum cutting temperature (OCT) compound (Miles Scientific, Naperville, IL) and quickly frozen in liquid nitrogen. Cryostat sections (100-µm thick) were stained overnight at 4°C with Cy3-conjugated monoclonal anti-smooth muscle α-actin (Sigma). Sections were counterstained with TO-PRO-3, washed with PBS, and viewed under the confocal microscope.

Data Analysis

Data are presented as means ± SE of n observations with P < 0.05 considered significant. The significance of differences between individual means was determined by two-tailed Student’s t-test. Differences among multiple means were evaluated by ANOVA corrected for multiple measures, where appropriate, and, when overall differences were detected, differences between individual means were evaluated post hoc using the Student-Newman-Keuls procedure.

RESULTS

The proliferative response of HASMCs during normoxic and hypoxic incubation was evaluated by cell counting (Fig. 1), 3H-thymidine incorporation (Fig. 2), and nuclear levels of S-phase proteins (Supplementary Fig. 1). A supplemental figure for this article is available online at the American Journal of Physiology Heart and Circulatory Physiology website. As shown in Fig. 1A, cell number is increased after incubation at 5% O2 for 48 h and after incubation at 3% O2 for 16 and 48 h. Incubation at 1% O2 for either 16 or 48 h reduced the total cell number. Similarly, in rat A7R5 smooth muscle cells, cell number increased 22.8 ± 6.5 and 31.4 ± 7.3% after incubation at 3% O2 for 16 and 48 h, respectively, (P < 0.05 vs. normoxic control, for both) and decreased by 38.8 ± 5.9 and 49.7 ± 8.6% after incubation at 1% O2 for 16 and 48 h, respectively (P < 0.05 vs. normoxic control, for both). Trypan blue was excluded (cells are viable) in 97.1 ± 0.9% of HASMCs after normoxic incubation and in 94 ± 0.4% after 16 h and 92.2 ± 0.6% after 48 h of incubation at 1% O2 (P < 0.05 vs. normoxic
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control values for both). HASMC viability did not differ between normoxic cells and cells incubated at 3% O2. HASMC cell numbers were decreased by incubation at 1% O2 in the absence and in the presence of PDGF after 16 (18.5 ± 4.6 and 19.7 ± 3.8% decrease from normoxic control values, respectively) and 48 h (24.4 ± 4.5 and 26.7 ± 2.9% decrease from normoxic control values, respectively, Fig. 1B). Incubation of HASMCs at 3% O2, either in the absence or presence of PDGF caused an increase in cell number at both 16 (20.3 ± 4.5 and 23.3 ± 4.8% increase from normoxic control values, respectively) and 48 h (35.6 ± 3.7 and 36.7 ± 4.8% increase from normoxic control values, respectively, Fig. 1C).

The effect of hypoxic incubation for up to 72 h on [3H]thymidine incorporation in HASMCs is illustrated in Fig. 2. Incubation at 1% O2 decreased (Fig. 2A), whereas 3% O2 increased (Fig. 2B) the rate of [3H]thymidine incorporation, reflecting the effects of these conditions on the rate of DNA synthesis. The effects of hypoxia on other markers of cell proliferation, Ki67, CDC6 (35), and MCM2 (36), are presented in Supplementary Fig. 1. The percentage of cells staining positive for Ki67 is decreased after incubation at 1% O2, whereas 3% O2 had the opposite effect (Suppl. Fig. 1A). Similarly, CDC6 protein is decreased and increased in cells incubated at 1 and 3% O2, respectively (Suppl. Fig. 1B), and MCM2 protein is reduced after incubation at 1% O2 (Suppl. Fig. 1C).

To determine whether hypoxia alters HASMC proliferation through a general effect on cell cycle progression or through mechanisms specific to a particular cell cycle phase or checkpoint, DNA content was quantified by PI staining (Fig. 3). After incubation at 1% O2, there is accumulation of G0/G1 phase cells and depletion of cells in S and G2/M phases (Fig. 3A), indicating a delay in G1/S transition. Conversely, incubation at 3% O2 results in a decrease in the percentage of cells in G0/G1 phase, an increase in S-phase cells, and a corresponding increase in the percentage of cells undergoing mitosis (Fig. 3B), suggesting acceleration of progression through the G1/S interphase. 94 ± 0.8% of cells remained viable (did not stain for either annexin V or PI) after normoxic incubation compared with 91.8 ± 0.7 and 83.7 ± 0.8% following incubation at 1% O2 for 16 and 48 h, respectively (P < 0.05 vs. corresponding normoxic control values for both). After incubation at 3% O2, the percentage of viable cells did not differ from the normoxic control value.

Since differences in cell number may reflect changes in the rate of cell death as well as proliferation, the prevalence of smooth muscle cell apoptosis after normoxic and hypoxic incubation was assessed in HASMCs by annexin V-PI staining and TUNEL (Fig. 4). The percentage of cells that stained with annexin V-FITC only (early apoptotic cells) is increased after incubation at 1% O2 and decreased at 3% O2 compared with the normoxic cells (Fig. 4A). Similarly, the percentage of TUNEL-positive cells is increased after incubation at 1% O2 (Fig. 4B).

Depolarization of the mitochondrial membrane both results from and contributes to impairment of cellular ATP synthesis and is an early event in apoptotic cell death. Figure 5 illustrates the effect of hypoxic incubation on the percentage of cells positive for JC-1 monomers (an index of mitochondrial membrane depolarization). In cells incubated at 1% O2 for 16 h, there is an increase in the percentage of monomer-positive cells, indicating mitochondrial membrane depolarization, and a further increase after 48 h (Fig. 5A). There is no change in the percentage of JC-1 monomer-positive cells after incubation at 3% O2 (Fig. 5B).

As shown in Fig. 6A, there is a decrease in cellular ATP concentration in HASMCs incubated for 16 h at 1% O2 and a further decrease after 48 h. Incubation at 3% O2 did not significantly alter cellular ATP concentration (Fig. 6B).

Gene expression profiling was used to identify oxygen-regulated genes and, hence, potential regulatory pathways. Complete gene expression data are presented in the online supplement (GEO accession no. GSE 4725). The fold changes from normoxic control values in the expression of genes with known pro- and antiproliferative and pro- and antiapoptotic roles, which demonstrated significant changes after hypoxic incubation, are presented in Table 1.

Figure 7 presents the effects of hypoxic incubation on nuclear levels of HIF-1α, p21, and p53. Protein concentrations were determined in these nuclear extracts by Bradford assay and appropriate volumes diluted with SDS loading buffer to
produce equal protein loading in all lanes. This was subse-
quently corroborated by full-lane densitometry of the Ponceau
red-stained membranes as described previously (66, 72, 78).
HIF-1
/H9251
levels are increased to a similar extent after incubation
at 1 and 3% O2. Levels of p21 and p53 proteins, inhibitory
regulators of cell cycle progression, are increased after incu-
bation at 1% O2 with no change after incubation at 3% O2.
Levels of TERT protein, a component of the telomerase com-
plex that is increased and enhances HASMC survival following
longer hypoxic epochs (46, 47), did not differ from
the normoxic control values after 16 and 48 h of incubation at
either 1 or 3% O2 (data not shown).

Vascular smooth muscle cell proliferation and cell death/
apoptosis were assessed in rats (Figs. 8 and 9) to evaluate the
effects of physiologically relevant levels of hypoxia on the
systemic vasculature in vivo. As shown in Fig. 8, exposure to
hypoxia increased the number of PI-staining cells (3.2 ± 1.9
vs. 18.6 ± 8.5% and 2.6 ± 1.5 vs. 12.7 ± 4.8% in aortae and
mesenteric arteries from normoxic vs. hypoxia-exposed rats,
P < 0.05 for both) with a corresponding increase in the number
of TUNEL-positive nuclei (2.7 ± 1.9 vs. 11.8 ± 7.5% and
1.8 ± 0.9 vs. 8.5 ± 4.4% in aortae and mesenteric arteries from
normoxic vs. hypoxia-exposed rats, P < 0.05 for both). Figure
9A shows that, in aortic and mesenteric artery sections from
hypoxia-exposed rats, there is an increase in the number of
BrdU-labeled cells compared with the normoxic control group.
Compared with normoxic animals, aortic and mesenteric artery
sections from hypoxia-exposed rats also demonstrate increased
medial nuclear density (Fig. 9B). Smooth muscle α-actin stain-
ing (Fig. 9C) confirms that the increase in cellularity reflects an
increase in medial smooth muscle.

**DISCUSSION**

We found the following in HASMCs in culture: 1) incubation
at 3% O2 enhances, whereas 1% O2 inhibits DNA synthe-
sis and the time-dependent increase in cell number; 2) incubation
at 1% O2 is associated with accumulation of cells in G1
phase of the cell cycle, whereas 3% O2 increased the percent-
age of cells in S and G2/M; 3) the prevalence of apoptosis is
increased after incubation at 1% O2; 4) cellular ATP levels are
reduced, and the mitochondrial membrane is depolarized after
exposure to 1% but not 3% O2; and 5) pro- and antiprolifera-
tive and pro- and antiapoptotic gene expression are tightly
coordinated to effect directionally opposed responses within a
narrow range of oxygen concentrations. In aortae and mesen-
teric arteries of rats breathing 10% O2 (arterial Po2 ≈ 40 Torr),
both smooth muscle cell proliferation and apoptosis are in-
creased.

Hypoxia has been reported to enhance proliferation in pul-
monary artery and aortic smooth muscle cells (13, 14, 16, 25,
43, 44, 54, 59). In apparent contradiction, others have observed
that hypoxic stress inhibits growth of these same cells (19, 20,
23). The results of the present study show that cell numbers,
[3H]thymidine incorporation, and biochemical markers of pro-
life (Ki67 (58), CDC6 (35), and MCM2 (36) protein
levels are increased and decreased after incubation at 3% O2 and 1% O2, respectively. We conclude that hypoxia may either enhance or inhibit HASMC proliferation depending on its severity. Rather than being due to experimental error or differences in cell origin, the discrepancy in previous observations, therefore, reflects fundamental differences in the nature of the cellular response elicited by varying degrees of hypoxic stress. The effect persists during and is additive to the effects of extrinsic stimulation with PDGF, suggesting that the responses are mediated by nonconvergent pathways. Total cell number, however, reflects effects on both proliferation and cell death, and differential modulation of these processes by hypoxia and/or PDGF could account for these findings without inferring independent signaling mechanisms.

Following incubation at 1% O2 we found that the percentage of cells in G1 phase of the cell cycle is increased, with a corresponding depletion of cells in G2/M and S phases, indicating a delay in progression through the G1/S interphase. In contrast, incubation at 3% O2 results in a decrease in the number of cells in G1 phase and an increase in DNA synthesis and in the percentage of mitotic cells, consistent with acceleration of the G1/S transition. The most frequently reported effect of hypoxia is delayed entry into S phase (19–23, 31, 57) as we observed in cells incubated at 1% O2. Our finding that enhanced proliferation in cells incubated at 3% O2 is also associated with an alteration in the rate of G1/S transition, albeit opposite in direction, is novel. It suggests that the bidirectional effects of hypoxia are integrated by events occurring at this checkpoint.

During transition from G1 to S phase, cyclin-dependent kinases (CDKs) phosphorylate the retinoblastoma protein, displacing the E2F-1 transcription factor and activating expression of S phase genes required for DNA synthesis (32). Activity of the CDKs is dependent on association with their respective cyclins and regulated by endogenous CDK inhibitors (e.g., p21 and p27). As shown in Table 1, genes whose products act at the G1/S transition are differentially regulated under the conditions studied. Both p53 and its transcriptional activation target p21, which inhibits G1/S transition, are in-
increased after incubation at 1% O2 and unchanged at 3% O2. Moreover, genes with known roles in modulating p53/p21 activity demonstrate patterns consistent with their expected functional effects. Expression levels of replication protein A1, which binds p53, preventing activation of p21 transcription (52) and components of the extracellular signal regulated kinase cascade, which stimulate assembly of the cyclinD-CDK4/6 complex, promoting G1/S progression and p27 degradation (75), are enhanced after incubation at 3% O2 and inhibited following incubation 1% O2. Replication factor C (activator 1)-1 (71), CDC6 (35), and MCM2 (36) are similarly affected. Conversely, expression of antiproliferative genes, inhibitor of growth family, member 1-like, which binds and enhances p53 activity (70), bone marrow morphogenetic protein 2, which induces Smad-mediated expression of p21 and p27 (76) and downregulates antiapoptotic Bcl-xl expression (79), and the dual specificity phosphatases 1 and 5, which deactivate ERKs (40), show the opposite pattern. Corresponding changes in tuberous sclerosis complex-1, a negative regulator of mRNA translation (42) (Table 1), suggest a mechanism by which the effects of hypoxia on protein and DNA replication may be coordinated.

The results of three independent assays (annexin V/PI, TUNEL, and mitochondrial membrane depolarization) and the changes in pro- and antiapoptotic gene expression (Table 1) in the present study indicate that the rate of apoptosis is increased in HASMCs incubated at 1% O2. The reduced cell number observed under this condition therefore reflects, in part, an enhanced rate of cell death. In fibroblasts and tumor cells, hypoxia of sufficient severity to cause ATP depletion impairs DNA repair, and the increase in apoptosis in this setting is considered a protective mechanism to prevent the accumulation of hypoxia-induced mutations (8, 10, 39). The increased rate of smooth muscle cell apoptosis during hypoxic epochs associated with reduced intercellular ATP concentration in the present study may serve a similar adaptive purpose. That hypoxia induces apoptosis at oxygen concentrations recorded in arteries affected by aneurismal dilatation and atherosclerotic plaques (51) and that hypoxemia in vivo increases smooth muscle apoptosis in the walls of systemic arteries support the suggestion that it may play a pathogenic role in arterial smooth muscle cell dysfunction (3) and loss in patients with cardiopulmonary disease.

Apoptosis may be triggered in response to stimuli extrinsic or intrinsic to the affected cell. Ligand binding to the TNF receptor superfamily such as Fas, through their association with the Fas-associated death domain (FADD) protein, results in assembly of the death-inducing signaling complex, which recruits and activates caspase-8. The intrinsic pathway is activated when mitochondrial membrane depolarization releases cytochrome C into the cytoplasm, where it binds apoptotic protease-activating factor-1 (Apaf-1), allowing it to activate pro-caspase-9. In the final common pathway caspase-9 (intrinsic) and caspase-8 (extrinsic) cleave and activate the effector protease, caspase-3 (53), p53, through interactions with Apaf-1 (63) and protective Bcl proteins, directly activates caspase-3 (17) and enhances permeability of the outer mitochondrial membrane (45). Complementary regulation by death inhibitors and the balance between prosurvival and proapoptotic Bcl-2 family members (1) superimpose an additional level of control. Our finding that mRNA expression (Table 1, microarray analysis) of components of both the extrinsic [caspase-8-associated protein 2 (Casp8) and FADD-like apoptosis regulator] and intrinsic [caspase recruitment domain protein 7, apoptosis-inducing serine/threonine kinase 17a and b, BMP2, Apaf-1, Bcl211 (Bcl2-antagonist of cell death), and p53 (22)] pathways, as well as caspase-3 that is itself oxygen regulated, therefore indicates that stimuli originating in the cellular microenvironment, as well as the intrinsic response, are important in hypoxic activation of the apoptotic program.

Resistance to apoptosis has been reported in lung fibroblasts, A7r5 cells, rat kidney proximal tubular cells, and rat pheochromocytoma PC12 cells during hypoxic incubation (2, 18, 37). In the present study, the percentage of cells staining positive for annexin V was decreased, and TUNEL showed a downward trend (P = 0.0932) in HASMCs incubated at 3% O2. Also the expression of antiapoptotic genes [Casp8 and FADD-like...
apoptosis regulator (27) and Bcl2-antagonist of cell death (49)] was increased following incubation at 3% O2 and 3% O2 on the expression of pro- and antiproliferative genes and pro- and antipoptotic genes in human aortic smooth muscle cells. Casp8, caspase-8-associated protein 2; FADD, Fas-associated death domain.

The mitochondria are the site of oxidative phosphorylation, however, and they must themselves consume ATP (64) to maintain the transmitochondrial membrane potential on which electron transport coupling depends (48). Failure of efficient ATP synthesis and, consequently, ion-motive ATPase activity ultimately result in depolarization of the membrane, further ATP depletion, Ca2+ influx, phospholipase and protease activation, and the release of apoptotic factors (12, 28, 50). Little information is available concerning the degree of hypoxia required to initiate programmed cell death, and it may vary among cell types (11). Our present results show that incubation of HASMCs at 1% but not 3% O2 causes mitochondrial membrane depolarization; therefore, the threshold between normal physiological functioning and cell destruction is exceptionally narrow in these cells, particularly compared with the range of oxygen concentrations to which they are normally exposed (41). Conflicts among previous reports are undoubtedly attributable, at least in part, to the lack of appreciation of this and the experimental rigor needed to separate these responses.

Nuclear levels of HIF-1α were elevated to a similar extent in HASMCs incubated at 3 and 1% O2, whereas the effects of these two conditions on HASMC proliferation were directionally opposed. This suggests that HIF-1-independent regulatory mechanisms predominate. It would be unusual, however, if the primary oxygen-sensing mechanism in mammalian cells were not involved in such an important response. Moreover, many cell cycle-associated genes that contain functional hypoxia regulatory elements and HIF-1-regulated pathways that both enhance cell survival (65, 77) and, conversely, increase apoptotic cell death (20, 24) have been identified. A more complex role that can be accounted for by changes in HIF-1α levels must therefore be proposed to reconcile these observations. Differences in mRNA expression of the HIF-1-regulated genes (29, 73) represented on the Affymetrix HG-U133A array in cells incubated at 1 and 3% O2 (see supplemental data, GEO accession no. GSE 4725) support this notion. This is not surprising since HIF-1α is subject to extensive posttranslational modification before nuclear translocation (4) and interacts with a multitude of coregulatory factors (20, 24, 30), offering many sites at which its function may be differentially affected by the two conditions.

During the prenatal period, the systemic circulation undergoes continuous restructuring in response to the changing requirements of the developing tissues. In the mature circulation, hypoxemia, due to cardiopulmonary disease, elicits responses that redistribute blood flow and enhance the capacity
for oxygen extraction (9, 33, 34). As the duration of hypoxia increases, systemic vascular smooth muscle and endothelial cell function are impaired (3, 67), limiting the efficacy of the acute responses. Concurrent structural remodeling thus plays an increasing role in maintaining the balance between oxygen delivery and metabolic demand (34). Our present findings indicate that this is facilitated by increases in the rates of both smooth muscle cell proliferation and death, a paradoxical state that will markedly enhance cell turnover. To achieve a degree of plasticity sufficient to enable the required structural change while avoiding the accumulation of mutations and malignant transformation or the formation of abnormal vascular structures, which exacerbate circulatory dysfunction, this process, however, must be tightly regulated. The results of the present study demonstrate that the difference between oxygen concentrations that enhance smooth muscle cell proliferation and those that impair cellular energy status and trigger cell destruction is correspondingly small.

Although both proliferation and apoptosis are enhanced in aortae and mesenteric arteries from hypoxia-exposed rats, the
The net effect is an increase in medial smooth muscle. Prolonged hypoxia of this severity results in a progressive loss of systemic arterial and arteriolar contractility (3, 68) with consequent impairment of the sympathetically mediated reflexes that regulate blood flow distribution (26). In this context, increased muscularity of the arterial wall can be viewed as a compensatory adaptation that preserves the capacity to regulate the systemic circulation. Vital organ function is highly intolerant of oxygen deprivation. Accordingly, mechanisms linking vascular cell turnover and the capacity for rapid structural change directly to oxygen concentration are required to avoid delays inherent in second messenger signaling. Our results indicate that pro- and antiproliferative and pro- and antiapoptotic gene expression are tightly coordinated to produce directionally opposed responses effected at the level of G1/S transition and involvement of both intrinsic and extrinsic apoptotic pathways.

Fig. 8. PI staining (A) of en face sections and TUNEL (B) in paraffin-embedded sections of normoxic and hypoxic (48 h) rat aorta and mesenteric artery (×40 magnification). Quantitative analysis (C) confirms increased cell death and apoptosis after hypoxic exposure; n = 6 rats per group. *P < 0.05 vs. corresponding normoxic control values.
Fig. 9. Immunohistochemical staining of incorporated bromodeoxyuridine (BrdU) (A) in paraffin-embedded sections of aorta and mesenteric artery from normoxic and hypoxia exposed (48 h) rats (×20 magnification). Double staining with TO-PRO-3 (B) and α-smooth muscle actin (C) in paraffin-embedded sections of normoxic and hypoxic (48 h) rat aorta and mesenteric artery shows increased cellularity and medial smooth muscle cell density after hypoxia (×40 magnification). Quantitative analysis (D) confirms increased proliferation and nuclear density, n = 6 rats per group. *P < 0.05 vs. corresponding normoxic control values.
Further definition of the individual roles of these regulatory mechanisms will be valuable in identifying therapeutic targets in conditions in which enhanced plasticity of the vasculature may be exploited to alleviate tissue oxygen deficiency or in which overexuberant remodeling interferes with normal cardiovascular function.

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