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SMG-1 kinase attenuates mitochondrial ROS production but not cell respiration deficits during hyperoxia

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Abstract

Purpose—Supplemental oxygen (hyperoxia) used to treat individuals in respiratory distress causes cell injury by enhancing the production of toxic reactive oxygen species (ROS) and inhibiting mitochondrial respiration. The suppressor of morphogenesis of genitalia (SMG-1) kinase is activated during hyperoxia and promotes cell survival by phosphorylating the tumor suppressor p53 on serine 15. Here, we investigate whether SMG-1 and p53 blunt this vicious cycle of progressive ROS production and decline in mitochondrial respiration seen during hyperoxia.

Materials and Methods—Human lung adenocarcinoma A549 and H1299 or colon carcinoma HCT116 cells were depleted of SMG-1, UPF-1, or p53 using RNA interference, and then exposed to room air (21% oxygen) or hyperoxia (95% oxygen). Immunoblotting was used to evaluate protein expression; a Seahorse Bioanalyzer was used to assess cellular respiration; and flow cytometry was used to evaluate fluorescence intensity of cells stained with mitochondrial or redox sensitive dyes.

Results—Hyperoxia increased mitochondrial and cytoplasmic ROS and suppressed mitochondrial respiration without changing mitochondrial mass or membrane potential. Depletion of SMG-1 or its cofactor, UPF1, significantly enhanced hyperoxia-induced mitochondrial but not cytosolic ROS abundance. They did not affect mitochondrial mass, membrane potential, or hyperoxia-induced deficits in mitochondrial respiration. Genetic depletion of p53 in A549 cells and ablation of the p53 gene in H1299 or HCT116 cells revealed that SMG-1 influences mitochondrial ROS through activation of p53.

Conclusions—Our findings show that hyperoxia does not promote a vicious cycle of progressive mitochondrial ROS and dysfunction because SMG-1-p53 signaling attenuates production of mitochondrial ROS without preserving respiration. This suggests antioxidant therapies that blunt ROS production during hyperoxia may not suffice to restore cellular respiration.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

Keywords

hyperoxia; mitochondrial dysfunction; p53; reactive oxygen species; SMG-1

Introduction

Elevated levels of oxygen (hyperoxia) are often used therapeutically to prevent hypoxemia in patients with respiratory insufficiency even though they can cause cell and tissue injury in the lung. One of the key mediators of oxygen toxicity is the accumulation of reactive oxygen species (ROS), which are produced in part by mitochondria.^[1–3] Mitochondria use oxygen to produce the majority of cellular energy in the form of ATP. Excess oxygen decreases mitochondrial respiration^[3,4] and thus may leave more oxygen available for reactions that overtly generate ROS. In addition to reacting with proteins and lipids, ROS induce oxidative lesions and single strand breaks in DNA within the first 24 h of hyperoxia exposure *in vitro*.^[5,6] The integrated relationship between mitochondrial ROS, respiration, and DNA damage, previously thought of as a vicious cycle, can contribute to the demise of the cell.^[7] In the case of hyperoxia, cells defend against oxygen-induced DNA damage by phosphorylating and stabilizing p53, which increases the transcription of genes such as the cyclin-dependent kinase inhibitor p21.^[8] p21 inhibits cell proliferation at the G1/S cell cycle checkpoint and delays cell death by preventing the loss of the antiapoptotic proteins Bcl-xL and Mcl-1.^[9,10]

The phosphatidylinositol 3-kinase-like kinases (PIKK) are responsible for phosphorylating p53 in response to genotoxic stress, including hyperoxia. Depending on the specific type of DNA damage, four of the six PIKKs can be activated: ataxia telangiectasia mutated (ATM), ataxia telangiectasia- and Rad3-related protein (ATR), DNA-dependent protein kinase (DNA-PK), and suppressor of morphogenesis of genitalia-1 (SMG-1). For example, both SMG-1 and ATM phosphorylate p53 following exposure to UV and ionizing radiation (IR) as well as in response to nuclear DNA double strand breaks induced by a restriction enzyme.^[11–13] These kinases are not completely redundant, however, as SMG-1 does not affect phosphorylation of the ATM target Chk2 induced by genotoxic stress^[11,12] and has greater *in vitro* kinase activity toward p53 than ATM.^[11] In addition, PIKKs such as ATM, DNA-PK, and SMG-1, maintain cellular homeostasis independent of their role in the DNA damage response.^[14–16] In A549 lung epithelial cells exposed to hyperoxia, SMG-1 is the PIKK mainly responsible for phosphorylating p53 serine 15 early during exposure, while ATM contributes to p53 phosphorylation later.^[12] ATM is likely responding to hyperoxia-induced DNA double strand breaks because it is activated after they are detected and not by overt mitochondrial dysfunction.^[3,17] A direct response to DNA damage by SMG-1 has not been observed. The temporal difference between SMG-1 and ATM activation during hyperoxia suggests distinct mechanisms determine not only their activation but also how they affect cellular outcomes.

SMG-1 was initially discovered for its role in mRNA surveillance, specifically the nonsense mediated mRNA decay (NMD) pathway.^[18,19] It participates in NMD by phosphorylating up-frameshift protein 1 (UPF1) in the SMG-1-UPF1-eRF1-eRF3 (SURF) complex, thus targeting mRNA containing a premature stop codon for degradation instead of translation.

[20] Independent of its role in NMD, SMG-1 also suppresses hypoxia response element-containing reporter activity,[21] blocks proteolysis of FLIP-L to prevent caspase-8 activation following TNF- α stimulation,[22] promotes telomere stability,[23] and prolongs the lifespan of *C. elegans* during starvation or paraquat exposure.[24] This seemingly disconnected ability of SMG1 to respond to and modulate a variety of stresses is consistent with it being found in the nucleus, mitochondria, and cytoplasm.[20,23,25]

Activation of SMG-1 within the first several hours of hyperoxia and before DNA double strand breaks are detected^[5,12] suggests a role in responding to early changes induced by oxygen availability or ROS. Because mitochondria regulate oxygen metabolism, cellular bioenergetics, and apoptosis, we hypothesized that SMG-1 is activated early during hyperoxia exposure to modulate and reestablish mitochondrial homeostasis. We provide novel evidence that SMG-1 serves to attenuate hyperoxia-induced mitochondrial ROS production without preserving or restoring mitochondrial respiration.

Materials and methods

Cell culture

A549 and H1299 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM; high glucose) with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μ g/mL streptomycin (Gibco, Carlsbad, CA) in 5% CO₂ at 37°C. The parental HCT116 human colon carcinoma cell line and isogenic cells lacking p53 (obtained from Dr. Bert Vogelstein) were cultured in McCoy's 5A medium (with glutamine, Gibco) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μ g/mL streptomycin. Cells were plated one day prior to exposure to room air (21% O₂, 5% CO₂) or hyperoxia (95% O₂, 5% CO₂) in a plexiglass chamber (Bellco Glass, Vineland, NJ) at 37°C. The 5% CO₂ is used to buffer pH in media containing bicarbonate.

siRNA transfection

Subconfluent cells were transfected with nontargeting (NT) siGENOME siRNA (Dharmacon, Lafayette, CO), 50 nM SMG1 siRNA (sense UACUUUGGCGCACAUACACTT, antisense GUGUAUGUGCGCCAAAGUATT, Dharmacon), 10 nM UPF1 siRNA (sense GAUGCAGUCCGCUCCAUTTT, antisense AAUGGAGCGGAACUGCAUCTT), or 10 nM p53 siRNA (sense CAGUCUACCUCGCCCAUATT, antisense UAUGGCGGGAGGUAGACUGTT) using Lipofectamine 2000 (Life Technologies, Eugene, OR) for 24 h, according to the manufacturer's instructions. Cells replated at subconfluency were exposed to hyperoxia the following day. RNA interference in each experiment was confirmed by immunoblotting.

Immunoblotting

Cells were collected by scraping with a rubber policeman in ice cold lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 25 mM sodium fluoride, 25 mM sodium β -glycerophosphate, 0.1 mM sodium vanadate, 0.1 mM PMSF, 0.2% Triton X-100, 0.3% IGEPAL CA-630, 0.1 μ g/mL pepstatin A, 1.9 μ g/mL aprotinin, and 2 μ g/mL leupeptin), sonicated twice for 15 s at 20% with a stepped microtip (Sonics Vibra Cell, Newtown, CT),

and diluted in 3X Laemmli Buffer (Laemmli at 1X contains 50 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 1% β -mercaptoethanol, and 0.1% bromophenol blue). Cellular fractions were obtained using the Cell Fractionation Kit-Standard (Abcam, Cambridge, MA) according to the manufacturer's instructions. Protein concentrations were determined using the BCA assay (Thermo Scientific, Rockford, IL), and lysates (10 μ g protein for subcellular fractions) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Pall Life Sciences, Pensacola, FL). After blocking with 5% nonfat milk in TBS-T (0.1% Tween-20), blots were incubated overnight at 4°C with primary antibody: SMG-1 (A300-393A), UPF1 (A301-902A) (Bethyl Laboratories, Montgomery, TX); p-p53 (Ser15) and Histone H3 (Cell Signaling Technology, Beverly, MA); p53 (DO-1) (Novus, Littleton, CO); Cytochrome *c* oxidase subunit 1 (Mitosciences, Eugene, OR); β -actin (Sigma). Membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies (Southern Biotech, Birmingham, AL) in 5% nonfat milk in TBS-T. Antibody complexes were detected using the ECL Plus Western Blotting Detection kit (GE Healthcare, UK) and visualized using x-ray film (Laboratory Products Sales, Rochester, NY). Blots analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) were normalized to β -actin within each experiment, as appropriate.

Measurement of mitochondrial mass and membrane potential

Adherent cells were stained in triplicate with 50 nM MitoTracker Green FM (Molecular Probes, Eugene, OR) in 1 $\times$ HBSS or 5 μ g/mL JC-1 (Molecular Probes) in 1 $\times$ PBS for 15 min at 37°C. Cells were washed with 1 $\times$ HBSS, trypsinized, and washed twice more before analysis. For JC-1 controls, cells were treated with 10 μ M carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP, Sigma) during staining. Using a BD LSRII flow cytometer (University of Rochester Medical Center Flow Cytometry Core, MitoTracker Green excitation 488 nm, emission 525 nm; JC-1 excitation 488 nm, green monomers detected at emission 525 nm and red aggregates at emission 610 nm), 10,000 events gated as singlets (FSC-A/SSC-A then SSC-H/SSC-W) were collected for analysis. FlowJo software v. 10 (FlowJo, Ashland, OR) was used to determine mean fluorescence intensity (MFI).

Measurement of mitochondrial respiration

The XF-96 Extracellular Flux Analyzer (Seahorse Biosciences, Billerica, MA) was used to measure the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). A549 cells on normal tissue culture plates were exposed to hyperoxia, trypsinized at the end of the exposure, and all treatments were replated on a polystyrene plate at 20,000 cells/well in 30 μ L in unbuffered DMEM pH 7.4 (1 mM sodium pyruvate, 4 mM L-glutamine, 25 mM dextrose) by centrifugation (40 $\times$ g for 1 s, reverse plate orientation, then 60 $\times$ g for 1 s, with slow acceleration and deceleration). After about 30 min, 120 μ L unbuffered DMEM was added to each well; the assay was initiated within 2 h of removal from hyperoxia. Basal respiration was measured before injection of 0.5 μ M FCCP (final concentration) to stimulate maximal OCR. Nonmitochondrial OCR was measured after injection with 1 μ M Antimycin A (Sigma) and subtracted from basal and maximal OCR measurements. Reserve capacity was calculated as the difference between maximal and basal OCR. Each experiment included 6–8 technical replicates per condition. For normalization, cells were lysed in 0.1 M

NaOH with 0.5% SDS and protein concentration was determined by BCA assay (Thermo Scientific).

Measurement of reactive oxygen species

Adherent cells were stained in triplicate with 5 μ M dihydroethidium (DHE) or 5 μ M MitoSOX Red (Molecular Probes) in $1 \times$ HBSS for 15 min at 37°C. Cells were washed with $1 \times$ HBSS, trypsinized, and washed twice more before analysis. HCT116 cells were trypsinized prior to staining with MitoSOX (15 min at 37°C) and were washed twice with $1 \times$ HBSS prior to analysis. Using a BD LSRII flow cytometer (excitation 532 nm, emission 575 nm), 10,000 events gated as singlets (FSC-A/SSC-A then SSC-H/SSC-W) were collected for analysis. FlowJo software v.10 was used to determine MFI.

Statistical analysis

Results represent at least three independent experiments and were graphed using Prism (GraphPad Software, La Jolla, CA). Statistical tests were done with JMP Software v. 11 (SAS, Cary, NC). A student's unpaired t-test was used to compare group means within a given treatment. A one-way ANOVA was used to compare group means between different treatment groups, with the Tukey-Kramer post hoc test to control for multiple comparisons.

Results

SMG-1 phosphorylates p53 on serine 15, a posttranslational modification that stabilizes p53 and promotes its transcriptional activity. A549 cells were transfected with NT or SMG-1 siRNA and then exposed to room air or hyperoxia for 12 and 24 h. Hyperoxia induced phosphorylation of p53 on serine 15 (p-p53 (Ser15)) in cells transfected with NT siRNA, and this was blunted in cells transfected with siRNA targeting SMG-1 (Figure 1A). Total p53 abundance concomitantly increased with hyperoxia exposure and was moderately decreased in cells transfected with SMG-1 siRNA. Likely due to the feedback mechanism between NMD factors,^[26] SMG-1 depletion increased the abundance of its cofactor UPF1 (Figure 1A). We also investigated whether hyperoxia altered the intracellular location of SMG1 or UPF1. Nuclear, mitochondrial, and cytoplasmic fractions were prepared from A549 cells exposed to room air or hyperoxia for 12h. Purity of the fractions were confirmed by immunoblotting for mitochondrial cytochrome *c* oxidase subunit I (COX1) and nuclear histone H3 (Figure 1B). SMG-1 was mainly detected in mitochondrial and cytoplasmic fractions of cells exposed to room air or hyperoxia (Figure 1B). UPF1 was detected predominately in the cytoplasmic fraction, while p-p53 (Ser15) was detected more in the nuclear than mitochondrial fraction following 12 h of hyperoxia (Figure 1B). Thus, SMG-1 is predominantly present in the cytoplasmic and mitochondrial regions of the cell.

As mitochondria are important for oxygen metabolism, the role of SMG-1 in regulating mitochondrial function was investigated. First, cells were transfected with NT or SMG-1 siRNA and stained with MitoTracker Green to assess mitochondrial mass. Decreased SMG-1 expression did not affect the moderate but statistically insignificant increase in MitoTracker Green fluorescence induced by 24 h of hyperoxia (Figure 2A). Also, the effects of SMG-1 depletion on mitochondrial membrane potential were assessed using JC-1, which

forms fluorescent red aggregates in highly polarized mitochondrial membranes or green monomers upon depolarization. Mitochondrial membrane potential was not significantly affected by SMG-1 siRNA. As a positive control for this assay, the uncoupling drug FCCP caused depolarization regardless of SMG-1 RNA interference (Figure 2B).

Next, mitochondrial respiration was measured using Seahorse XF technology. SMG-1 depletion significantly decreased basal OCRs in room air conditions, but did not significantly affect the hyperoxia-induced decrease in OCR at 12 and 24 h (Figure 2C). Maximal respiration (Figure 2D) and reserve capacity, or the ability of cells to respond to mitochondrial uncoupling (Figure 2E), decreased over time in hyperoxia but was not significantly affected by SMG-1 depletion. The ECAR, which partially reflects lactic acid produced by glycolysis, was significantly decreased in room air but not in hyperoxia when SMG-1 expression was decreased (Figure 2F). Thus, SMG-1 depletion does not significantly affect hyperoxia-induced changes in bioenergetics as defined by changes in OCR, mitochondrial reserve capacity, or ECAR.

Because ROS are thought to drive cellular oxygen toxicity, we next determined the ability of SMG-1 to protect against ROS accumulation. Cells transfected with NT or SMG-1 siRNA were exposed to hyperoxia and then stained with fluorescent probes that mainly, but not exclusively, react with superoxide.^[27] While hyperoxia increased DHE staining at 24 h as expected, SMG-1 siRNA did not significantly affect DHE compared to NT siRNA (Figure 3A). In contrast, fluorescence of the mitochondrial-targeted form of DHE, MitoSOX, was significantly greater in cells transfected with SMG-1 siRNA than NT siRNA at both 12 and 24 h of hyperoxia (Figure 3B). Prolonging hyperoxia exposure to 48 h further increased DHE and MitoSOX fluorescence, which SMG-1 depletion significantly augmented (NT v. SMG-1: DHE 2.38 ± 0.12 v. 3.08 ± 0.25 , MitoSOX 4.29 ± 0.24 v. 8.53 ± 0.65). Therefore, SMG-1 depletion amplified the level of mitochondrial ROS induced by hyperoxia exposure.

To determine if SMG-1 regulation of mitochondrial ROS is dependent on its role in NMD or its kinase activity toward p53, the involvement of UPF1 and p53 was investigated. While transfection with UPF1 siRNA increased the abundance of SMG-1, there was a decrease in p-p53 (Ser15) at 24 h, as previously observed,^[13] and a significant increase in MitoSOX fluorescence under room air and hyperoxic conditions (Figure 4A, B). Similarly, cells transfected with p53 siRNA had greater MitoSOX fluorescence in room air in hyperoxia ($P = .11$) (Figure 4C, D). The p53-deficient H1299 lung cancer cell line was also used to test whether SMG-1 depends on p53 to regulate mitochondrial ROS. SMG-1 depletion increased MitoSOX by 1.4 fold in both room air- and hyperoxia-exposed cells ($P = .054$ in hyperoxia), indicating SMG-1 was influencing production of mitochondrial-derived ROS independent of whether the cells were cultured in room air or hyperoxia (Figure 5A). Moreover, the compensatory increase in UPF1 abundance was not as great as that observed in A549 cells (compare Figure 5B and Figure 1A), suggesting that loss of p53 or other genetic differences between A549 and H1299 cells mediated the SMG-1 effects.

Because differences between nonhomologous cell lines could account for the different responses to hyperoxia-induced ROS with SMG-1 depletion, wild type and isogenic p53-deficient HCT116 cells were also used to elucidate the relationship between SMG-1 and

mitochondrial ROS production. Transfection with SMG-1 siRNA moderately decreased SMG-1 protein abundance in both wild type and p53 deficient cells but did not extensively affect p-p53 (Ser15) in wild type cells (Figure 6A). SMG-1 siRNA increased the abundance of UPF1 in wild type but not p53 deficient cells (Figure 6A). Decreased SMG-1 expression augmented the hyperoxia-induced increase in MitoSOX fluorescence in wild type cells (Figure 6B); however, MitoSOX fluorescence in room air and hyperoxia was unaffected in p53 deficient cells transfected with SMG-1 siRNA (Figure 6C). Therefore, the effect of SMG-1 depletion on hyperoxia-induced mitochondrial ROS is dependent on the presence of p53.

Discussion

In the midst of a complex cellular response to hyperoxia, SMG-1 phosphorylates p53 to activate pathways that promote cell survival. Because SMG-1 can regulate several aspects of cellular homeostasis, this study tested the hypothesis that SMG-1 is responsible for modulating the production of ROS and mitochondrial function in hyperoxia. While SMG-1 depletion did not appear to affect the changes in mitochondrial respiration seen during hyperoxia exposure, SMG-1 depletion was found to increase mitochondrial ROS. These results were replicated by decreasing the expression of UPF1 and p53 in A549 cells and, along with results from two other cell lines, suggests that activation of the SMG-1-p53 pathway is responsible for repressing hyperoxia-induced mitochondrial ROS formation. Taken together, these findings show that early SMG-1 activation during hyperoxia is important for attenuating oxidative stress but does not protect against decreases in mitochondrial respiration.

SMG-1 protein has been detected throughout the cell and may contribute to maintaining homeostasis through different mechanisms in each location. Here, SMG-1 was detected predominantly in mitochondrial and cytoplasmic fractions, and to a lesser extent in the nucleus. Exposure to hyperoxia did not change this distribution. UPF1, an RNA helicase that is both a regulator and a substrate of SMG-1 kinase activity,^[28] was predominantly detected in the cytoplasmic fraction where NMD occurs.^[20] The NMD network is complex, in that the removal of one protein in the complex can affect the abundance of other proteins.^[26] Up-regulation of SMG-1 upon depletion of UPF1 was not enough to maintain p-p53 (Ser15). Because UPF1 is required for SMG-1 to phosphorylate p53 during hyperoxia, it is likely that SMG-1 performs this post-translational modification in the cytoplasm before p-p53 (Ser15) is trafficked to the nucleus for its transcriptional activity^[29] or to mitochondria for an unidentified purpose. There is no evidence in the literature to our knowledge that indicates the NMD process alone activates p53, suggesting a unique role for SMG-1 activation in hyperoxia.

Because mitochondria are responsible for generating ATP used to repair oxidative damage yet become dysfunctional during hyperoxia, we hypothesized that SMG-1 could regulate mitochondrial respiration. Our findings suggest that SMG-1 promotes mitochondrial respiration under basal conditions but is not involved in the changes induced by hyperoxia. The effects on respiration do not appear to be due to differences in mitochondrial mass nor does the decreased respiration affect the mitochondrial membrane potential. Under room air

conditions, the availability of substrates required for oxidative phosphorylation and glycolysis may be affected by an altered proteome caused by decreased NMD or by a lack of posttranslational modifications performed by SMG-1. While phosphorylation of proteins such as pyruvate dehydrogenase, NADH dehydrogenase, and cytochrome *c* oxidase have been shown to modulate metabolism,^[30] the phospho-proteome of SMG-1 is not well defined. Nonetheless, SMG-1 is involved in regulating cellular metabolism in physiologic conditions but does not protect against mitochondrial dysfunction induced by hyperoxia exposure.

Hyperoxia-induced toxicity is thought to be mediated in large part by increased ROS levels.^[31–33] The results presented here show that genetic manipulation of SMG-1 could specifically alter mitochondrial ROS. While increased MitoSOX fluorescence could occur due to increased mitochondrial membrane potential,^[27] SMG-1 siRNA did not significantly affect the fluorescence ratio of JC-1, indicating that SMG-1 deficiency causes an accumulation of mitochondrial ROS during hyperoxia by a mechanism independent of membrane potential. This supports studies that indirectly show that SMG-1 deficiency causes oxidative stress^[11,25] and that SMG-1 is important for a pro-survival response to oxidative stress.^[21,24,25]

While SMG-1 localization in mitochondria could make direct regulation of the organelle possible, the results presented here imply mitochondrial ROS can be attenuated by SMG-1 kinase activity toward p53. In addition to the results in A549 and HCT116 wild type cells, the constant amplification of mitochondrial ROS observed with SMG-1 depletion in p53-deficient H1299 cells, and lack thereof in HCT116 p53^{−/−} cells, suggests a p53-dependent mechanism. Because SMG-1 depletion increased hyperoxia-induced mitochondrial ROS in the presence of p53, SMG-1 may function to reassign the role of p53 from prooxidant to antioxidant. The transcriptional profile of p53 early during hyperoxia might involve upregulation of antioxidant genes in addition to cell cycle and survival genes such as p21^[12,34]; however, SMG-1 depletion did not significantly affect the abundance of the protein responsible for detoxifying mitochondrial superoxide, MnSOD (data not shown). Independent of p53, SMG-1 can phosphorylate cdc25c and thus allow Cdk2 to activate the G1/S checkpoint following IR.^[35] In contrast to the protection mediated by SMG-1 following hyperoxia or IR,^[11] one study showed that Smg1 inactivation could prolong the lifespan of *C. elegans* during paraquat exposure independent of NMD but partially dependent on cep1/p53.^[24] These studies support multifaceted modes for how SMG-1 may depend on the presence of p53 to regulate and respond to oxidative stress.

The significant increase in mitochondrial ROS induced by SMG-1 depletion during hyperoxia was not associated with enhanced deficits in respiration, implying that hyperoxia affects mitochondrial respiration by a mechanism independent from how SMG-1 regulates ROS. This break in the “vicious cycle” may explain why antioxidant therapies in premature infants have not been completely efficacious in preventing lung disease.^[36–38] For example, treating premature infants with recombinant CuZnSOD did not prevent the development of bronchopulmonary dysplasia at 4 weeks of age or 36 weeks postmenstrual age, but it improved pulmonary outcomes assessed at 1 year of age.^[36] Other changes in mitochondrial

homeostasis, such as bioenergetics, may also contribute to the acute side effects of oxygen therapy.

It remains to be determined whether the current findings are specific for hyperoxia-induced oxidative stress or how cells respond to any type of oxidative stress. They are, however, likely to represent a response of proliferating cells to oxidative stress because hyperoxia-induced p53 signaling occurs in proliferating but not quiescent A549 cells.^[39] A549 and H1299 carcinoma cells are considered to be models of alveolar epithelial type II cells, which are considered targets of hyperoxia yet not easily manipulated for primary cell culture experiments because they transdifferentiate into alveolar type I cells. Because adult type II cells are relatively senescent unless there is injury to the lung, our findings with A549 and H1299 cells may reflect how proliferating neonatal type II cells or other types of adult adenocarcinomas respond to hyperoxia. Less proliferative cells, such as small airway lung epithelial cells, might not experience as much hyperoxia-induced mitochondrial ROS and thus not require SMG-1-p53 pathway activation (data from primary human small airway epithelial cells not shown) as occurs in proliferating cells. Regardless of whether SMG-1 regulation of mitochondrial ROS occurs in a specific lung cell type, the results reveal a disconnect in the vicious cycle of mitochondrial dysfunction.

In summary, SMG-1 activation early during hyperoxia exposure is important for repressing the accumulation of mitochondrial ROS that contributes to oxygen toxicity. Despite reducing the production of toxic ROS, SMG-1 does not influence oxygen-dependent loss in mitochondrial respiration. This may help explain why administration of the antioxidant CuZn-superoxide dismutase (SOD) was partially protective to preterm infants receiving supplemental oxygen.^[36] Although CuZn-SOD reduced the incidence of lung disease at 1 year of age, it did not reduce the incidence of bronchopulmonary dysplasia or mortality, which may reflect failure to restore mitochondrial respiration. In order to provide better treatment to individuals requiring supplemental oxygen, more research is needed to understand specific mechanisms causing SMG-1 activation and its ability to block ROS production, while other studies are needed to understand how mitochondrial respiration can be preserved during exposure.

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Figure 1.

SMG-1 phosphorylates p53 during hyperoxia and is enriched in cytoplasmic and mitochondrial fractions. (A) A549 cells transfected with nontargeting or SMG-1 siRNA were exposed to 21% (RA) or 95% (O₂) oxygen for 12 and 24 h, and protein was collected as whole cell lysate. (B) Cytoplasmic (1), mitochondrial (2), and nuclear (3) fractions were collected from untransfected A549 cells exposed to 12 h RA or O₂. Lysates were immunoblotted as indicated, with cytochrome *c* oxidase subunit 1 (COX1) and histone H3 showing enrichment of mitochondrial and nuclear proteins in (B), respectively. Western blots are representative of at least three experiments, with densitometric means shown below each blot.

Figure 2.

Decreased SMG-1 expression does not affect hyperoxia-induced changes in mitochondrial mass, membrane potential, or respiration. Cells transfected with nontargeting (NT) or SMG-1 siRNA were exposed to 21% (RA) or 95% (O₂) oxygen for 12 and 24 h. (A) MitoTracker Green mean fluorescence intensity (MFI) was analyzed by flow cytometry and normalized to RA NT within each experiment. (B) The ratio of red:green fluorescence of JC-1 represents the mitochondrial membrane potential. As a positive control, RA cells were stimulated with FCCP during JC-1 staining. (C–F) Cells were replated on XF96 plates immediately before the assay. After measuring basal respiration, the maximal oxygen consumption rate (OCR) was stimulated with FCCP and inhibited with antimycin A. Respiration rates were normalized to the mean μg of protein per group. (C) Basal OCR and (D) maximal OCR were calculated by subtracting nonmitochondrial OCR. (E) Reserve capacity was calculated as the difference between basal and maximal OCR. (F) Baseline extracellular acidification rates (ECAR) were measured in the same experiments as C–E. Data are presented as mean ± SEM (*O₂ compared to RA, # SMG-1 siRNA compared to NT, $P < .05$ ANOVA with Tukey-Kramer post hoc, $n = 3–5$).

Figure 3. SMG-1 depletion increases mitochondrial ROS in hyperoxia. A549 cells transfected with nontargeting (NT) or SMG-1 siRNA were exposed to 21% (RA) or 95% (O₂) oxygen for 12 and 24 h and then stained with (A) 5 μ M DHE or (B) 5 μ M MitoSOX for analysis by flow cytometry. Mean fluorescence intensity (MFI) was normalized to RA NT within each experiment. Data are presented as mean $\pm$ SEM (*compared to RA, #compared to NT, $P < .05$ ANOVA with Tukey-Kramer post hoc, $n = 4$).

Figure 4.

Decreased UPF1 and p53 expression induces mitochondrial ROS. A549 cells transfected with nontargeting (NT), UPF1, or p53 siRNA were exposed to 21% (RA) or 95% (O₂) oxygen for 24 h. (A, C) Cells were stained with 5 μM MitoSOX and analyzed by flow cytometry. Data are presented as mean ± SEM (* $P < .05$, ** $P < .01$ as compared to NT, Student's unpaired t-test, A, $n = 3$; C, $n = 4$). (B, D) Whole cell lysates from the same experiments as in (A) and (C) were collected and immunoblotted for the indicated proteins (B, $n = 3$; D, $n = 2$). In (B), lysates were immunoblotted on the same membrane but re-grouped for publication.

Figure 5.

Decreased SMG-1 expression induces mitochondrial ROS in H1299 cells. H1299 cells transfected with nontargeting (NT) or SMG-1 siRNA were exposed to 21% (RA) or 95% (O₂) oxygen for 24 h. (A) Cells were stained with 5 μ M MitoSOX and analyzed by flow cytometry. Data are presented as mean $\pm$ SEM (* P < .05, ** P < .01 as compared to NT, Student's unpaired t-test, n = 3). (B) Whole cell lysates from one of the same experiments were collected and immunoblotted for the indicated proteins.

Figure 6.

Decreased SMG-1 expression enhances mitochondrial ROS in wild type but not p53 deficient cells exposed to hyperoxia. HCT116 wild type (wt) and p53 deficient (p53^{-/-}) cells transfected with nontargeting (NT) or SMG-1 siRNA were exposed to 21% (RA) or 95% (O₂) oxygen for 24 h. (A) Whole cell lysates were collected and immunoblotted for the indicated proteins. A representative experiment is shown, with densitometric means shown below each blot. (B, C) Cells were stained with 5 μ M MitoSOX and analyzed by flow cytometry. Data are presented as mean $\pm$ SEM (* compared to RA, # compared to NT, $P < .05$ ANOVA with Tukey-Kramer post hoc, $n = 3$).