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Nuclear factor κ B1/RelA mediates inflammation in human lung epithelial cells at atmospheric oxygen levels

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Abstract

Oxygen levels range from 2–9% *in vivo*. Atmospheric O₂ levels (21%) are known to induce cell proliferation defects and cellular senescence in primary cell cultures. However, the mechanistic basis of the deleterious effects of higher O₂ levels is not fully understood. On the other hand, immortalized cells including cancer cell lines, which evade cellular senescence are normally cultured at 21% O₂ and the effects of higher O₂ on these cells are understudied. Here we addressed this problem by culturing immortalized human bronchial epithelial (BEAS-2B) cells at ambient atmospheric, 21% O₂ and lower, 10% O₂. Our results show increased inflammatory response at 21% O₂ but not at 10% O₂. We found higher RelA binding at the NF- κ B1/RelA target gene promoters as well as upregulation of several pro-inflammatory cytokines in cells cultured at 21% O₂. RelA knockdown prevented the upregulation of the pro-inflammatory cytokines at 21% O₂, suggesting NF- κ B1/RelA as a major mediator of inflammatory response in cells cultured at 21% O₂. Interestingly, unlike the 21% O₂ cultured cells, exposure of 10% O₂ cultured cells to H₂O₂ did not elicit inflammatory response, suggesting increased ability to tolerate oxidative stress in cells cultured at lower O₂ levels.

Keywords

Oxygen tension; Oxidative stress; Inflammation; Lung epithelial cells; Reactive oxygen species

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Conflict of Interest:

None

Introduction

Oxygen (O₂) partial pressure in various tissues and organs is considerably different from that of inhaled ambient atmospheric O₂ (21%). Depending on the degree of circulation to a particular tissue or organ, the O₂ levels in the body ranges between 2 and 9% (Brahimi-Horn and Pouyssegur, 2007). Accumulating evidence suggests lower growth and survival rates of cells cultured at 21% O₂, compared to the cells cultured at lower physiological levels (Caldwell et al., 2001; Carswell et al., 2000; Estrada et al., 2012; Fehrer et al., 2007; Forsyth et al., 2006; Garreta et al., 2014). Mouse embryonic fibroblasts (MEFs) cultured at 3% O₂ showed increased life span, decreased cellular senescence and lower DNA damage, compared to the cells cultured at 21% O₂ (Parrinello et al., 2003). In addition, primary cells grown at 5% O₂ showed higher glucose utilization and produced lower inhibitory metabolites (Estrada et al., 2012; Folmes et al., 2011).

On the other hand, growing primary cells at 21% O₂ increased intracellular ROS and decreased antioxidant levels, indicating oxidative stress (Ames et al., 1993; Atkuri et al., 2007). Several adverse effects including chromosomal aberrations (Forsyth et al., 2006; van Gent et al., 2001), cellular senescence (Betts et al., 2008) and loss of viability (Boregowda et al., 2012) have been reported at 21% O₂. In addition, macromolecular damage including protein carbonylation, fatty acid oxidation (Konigsberg et al., 2013) and DNA damage (Estrada et al., 2012; Ho et al., 2007) occurs at 21% O₂. Previous studies have observed alterations in transcriptional regulation at 21% O₂. Primary T-cells displayed increased expression of genes involved in cell death, cellular repair and stress response (Haddad et al., 2004) and human peripheral blood mononuclear cells (PBMCs) expressed characteristic cell surface markers typically seen during infection, at 21% O₂ (Atkuri et al., 2005; Atkuri et al., 2007). Primary human mesenchymal stem cells (MSCs) cultured at 21% O₂ showed p53 dependent inhibition of growth and differentiation, mitochondrial ROS generation, reduced cell viability and inhibition of cell proliferation (Boregowda et al., 2012).

At lower O₂ levels activation of HIF1- α mediated glycolytic function, which decreases mitochondrial oxygen consumption and reactive oxygen species (ROS) generation has been suggested to have a protective effect on primary human MSCs (Estrada et al., 2012). However, the mechanistic basis of the adverse effects of higher O₂ levels is not fully understood. Moreover, the current understanding of the favorable effects of lower O₂ levels is largely derived from work on primary cells, which exhibit impaired growth and survival at 21% O₂ (Alaluf et al., 2000; Atkuri et al., 2005; Boregowda et al., 2012). Unlike primary cells, the immortalized cell lines do not exhibit apparent growth difficulties at 21% O₂, most likely due to their ability to evade senescence (Shay et al., 1993; Shay and Wright, 2005). Therefore, usage of 21% O₂ is a standard practice for *in vitro* cell culture experiments. However, the effects of non-physiological, higher O₂ levels on these cells are understudied. Given the number of studies that are carried out on cells cultured at 21% O₂, it is critically important to obtain a better understanding of the cellular processes and pathways affected at higher than physiological levels of O₂.

To accomplish this, we investigated the effects of atmospheric (21%) and lower (10%) O₂ levels on the immortalized, non-cancerous human bronchial epithelial BEAS-2B cells in

both short- and long-term cultures. Our studies unearthed NF- κ B1/RelA mediated activation of pro-inflammatory cytokines at 21% O₂ but not at 10% O₂. Knocking down RelA reversed this effect. Moreover, we show that the cells grown at 10% O₂ display increased ability to tolerate external oxidative stress induced by H₂O₂ exposure, compared to the cells grown at 21% O₂.

Materials and Methods

Cell culture and treatments

Immortalized human bronchial epithelial (BEAS-2B) cells were cultured as previously reported (Jose et al., 2014) in Dulbecco's modified Eagle's medium (DMEM) (Cellgro), supplemented with 1% (vol/vol) penicillin–streptomycin and 10% (vol/vol) FBS (Atlanta Biologicals) at 37 °C. For 21% O₂ cultures, the cells were incubated in a standard incubator (NuAire NU-8700 AutoFlow water jacketed CO₂ incubator). For 10% O₂ cultures, the culture medium was pre-equilibrated at 10% O₂ overnight. The cells growing at 21% O₂ for several generations were then transferred to the pre-equilibrated culture medium and were incubated at 10% O₂ in a Panasonic MCO-5M-PA O₂/CO₂ incubator equipped with zirconia sensor and automatic O₂ cylinder switch over system for constant monitoring and maintenance of O₂ levels. CO₂ levels were maintained at 5% in both the conditions. The cells were cultured at 10% or 21% O₂ for 72 h (acute) or 3 weeks (chronic). For H₂O₂ exposure, BEAS-2B cells were treated with 150 μ M H₂O₂ for 72 h. For H₂O₂ treatment of cells cultured at 10% O₂, the cells were incubated at 10% O₂ for 12 h prior to H₂O₂ addition.

Measurement of reactive oxygen species (ROS) and antioxidant levels

The cells were trypsinized using 0.05% trypsin-EDTA solution (Gibco) and resuspended in growth medium. The cells (1.0×10^6) were dispensed into 5 mL polystyrene tubes, pelleted and incubated in the dark for 30 minutes at 37°C with 25 μ M 5-(and-6)-chloromethyl-2m,7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes Life Technologies). The cells were then washed twice with PBS. Fluorescence emitted by 100,000 live cells was quantified using a Miltenyi Biotec MACSQuant Analyzer. The peak excitation wavelength for oxidized H₂DCFDA was 488 nm and emission was 525 nm.

For measuring antioxidant levels in 72 h cultures grown at different O₂ levels, BEAS-2B cells were seeded in triplicates at a density of 1.5×10^3 cells/well in 96 well plates. The ratio between reduced (GSH) and oxidized glutathione (GSSG) was evaluated using Promega GSH/GSSG-glo luminometric assay kit (V611), according to manufacturer's protocol. For antioxidant measurement in 3-week cultures, on day 18, the cells were trypsinized and seeded in 96 well plates, at a seeding density of 1.5×10^3 cells/well. On day 21 the GSH/GSSG ratio was measured using Promega GSH/GSSG-glo luminometric assay kit. Statistical significance was evaluated using nonparametric t-test.

Cell proliferation

Rate of cell proliferation was measured using MTT assay. For this, 1.5×10^3 BEAS-2B cells were seeded in triplicates in 96 well plates and cultured for 72 h. Cell proliferation rate was

measured at 0, 24, 48 and 72 h time points using Promega CellTiter 96 non-radioactive cell proliferation assay kit (G4001), according to manufacturer's protocol. Statistical significance was evaluated using nonparametric t-test.

RNA Isolation and Quantitative PCR

Total RNA was isolated from cells using RNeasy kit (Qiagen) according to manufacturer's instructions. Quantitative PCR (qPCR) analysis was performed using Taqman Universal PCR Master Mix (Applied Biosystems) or FastStart Universal SYBR Green Master Mix (Roche) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Statistical significance of all qPCR results was evaluated using t-test ($p < 0.05$ (*); $p < 0.005$ (**); $p < 0.0005$ (***)).

RNA-Seq library preparation and data analysis

Gene expression analysis was carried out using RNA-Seq analysis of two biological replicates. RNA-Seq libraries were prepared using Illumina TruSeq RNA Sample Preparation Kit (RS-122-2002), according to the manufacturer's instructions. RNA-Seq data analysis was performed using BioWardrobe Experimental Management System (Kartashov and Barski, 2015). Briefly, the raw Fastq sequence files were aligned to the human genome (hg19) using STAR (Dobin et al., 2013) (version 2.4.0) using default parameters; multi hits removed) with a known reference annotation gtf file from RefSeq. Gene expression levels were quantified as reads per kilobase of exon per million fragments mapped (RPKM) using the BioWardrobe algorithm (Kartashov and Barski, 2015). Genes with RPKM > 1 in at least one experimental condition were considered as expressed. Differential gene expression was calculated using DESeq2 (Love et al., 2014). Genes that show 1.5 fold up- or down-regulation between the conditions compared, along with adjusted p -value < 0.1 were considered as differentially expressed and used for further analysis. The RNASeq data was deposited in the Gene Expression Omnibus (GEO) under the accession number GSE68378.

Gene ontology (GO) and canonical pathway analysis

Gene ontology (GO) biological functions and canonical pathways of the differentially expressed genes were identified using ToppGene Suite (Chen et al., 2009), which calculates functional enrichments using ToppGene knowledgebase (Chen et al., 2009). Hypergeometric distribution with Bonferroni correction ($p < 0.05$) is used for determining statistical significance. The GO terms ($p < 0.05$) are visualized using REVIGO visualizer (Supek et al., 2011), which uses SimRel algorithm to cluster GO terms based on semantic similarity, uniqueness and significance.

Motif analysis

Transcription factor binding motifs overrepresented at the promoters of the differentially expressed genes were identified by analyzing their sequences (-250 to +50 bp around the transcription start sites [TSS]) using PScan (Zambelli et al., 2009). The motifs were compared to known transcription factor binding sequences contained within the JASPAR database (Mathelier et al., 2014; Sandelin et al., 2004; Vlieghe et al., 2006) to identify the enriched motifs (FDR $p < 0.05$).

Identification of upstream regulators

Upstream regulator analysis (URA) (Kramer et al., 2014) was used to identify the upstream regulators potentially involved in generating the gene expression profiles observed in cells cultured at 21% O₂. The enrichment scores (Fisher's exact test p-values) along with the z-scores were used for ranking the top upstream regulators. The URA mechanistic networks generated by linking plausible directional networks from these upstream regulators based on Ingenuity Knowledge Base (Kramer et al., 2014) is displayed in figures 3D and 4I.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described earlier (Barski et al., 2007; Jose et al., 2014). Briefly, the cells were cross-linked with 1% formaldehyde for 10 min at 25 °C and sonicated to obtain 200- to 500-bp fragments. ChIP was performed using ChIP-grade antibodies against RelA (Abcam, ab7970).

RelA knockdown

BEAS-2B cells were transfected with RelA siRNAs (Dharmacon ON-TARGETplus SMARTpool [L-003533-00-0005]) or control oligonucleotides (Dharmacon [D-001810-10-05]) using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. The cells were collected after 72 h and RelA knockdown efficiency was evaluated by assessing RelA mRNA (qPCR) and protein levels (western blotting). To investigate the effect of 10% and 21% O₂ on RelA depleted cells, the RelA knockdown cells and the cells transfected with the control siRNAs were incubated at 10% or 21% O₂ for further 72 h. At the end of 72 h incubation, RelA knockdown efficiency was examined again by assessing the mRNA and protein levels.

Results

21% O₂ induces oxidative stress in cultured cells

To gain insight into the cell's response to atmospheric and lower O₂ levels, we cultured BEAS-2B cells at 21% and 10% O₂, respectively, for 72 h. First, to assess the extent of reactive oxygen species (ROS) generation in cells grown at different O₂ levels, we determined the percentage of cells that stained with ROS marker 5-(and-6)-chloromethyl-2m,7'-dichlorodihydrofluorescein diacetate (H₂DCFDA). The cells grown at 21% O₂ exhibited significant increase in ROS compared to the cells grown at 10% O₂ (Fig. 1A). We next assessed the levels of ROS scavenging endogenous antioxidants by quantifying the GSH/GSSG ratio. The cells cultured at 21% O₂ displayed decrease in the antioxidant (GSH) levels indicating accumulation of oxidized glutathione (GSSG) (Fig. 1B). The increased ROS and decreased antioxidants suggest elevation of oxidative stress at 21% O₂. In addition, we detected time-dependent decline in the rate of cell proliferation at 21% O₂ (Fig. 1C).

Pro-inflammatory pathways are upregulated at 21% O₂

To determine the influence of varying O₂ levels on transcriptional regulation, we used RNA-Seq to profile gene expression in BEAS-2B cells cultured at 21% and 10% O₂. In the cells

grown at 21% O₂, we identified 1.5 fold up- and down- regulation of 214 and 301 genes, respectively, compared to the 10% O₂ cultured cells. To categorize the biological processes that are potentially impacted by higher O₂ levels, we performed gene ontology (GO) analysis of the differentially expressed genes. The top biological process GO terms associated with the genes upregulated in 21% O₂ include inflammatory signaling, immune system processes and cytokine production (Fig. 2A), suggesting inflammation as a major consequence of culturing cells at higher O₂ levels. On the other hand, GO processes related to cell proliferation, focal adhesion and cell migration are enriched in the genes downregulated at 21% O₂ (Fig. 2B), suggesting impaired cellular growth and proliferation at 21% O₂.

In order to identify the pathways that are associated with the observed biological functions, we performed gene list enrichment analysis for canonical pathways using Toppgene knowledgebase (Chen et al., 2009). Inflammatory response and cellular stress associated pathways including TNF, NOD-like receptor signaling and ATF2 transcription factor network pathways were upregulated at 21% O₂ (Fig. 2C), further signifying inflammation as an important outcome of exposure to atmospheric O₂ levels. Genes associated with cell proliferation and cell-cell communication were downregulated at 21% O₂ (Fig. 2D), potentially contributing to the cell proliferation defects observed at 21% O₂ (Fig. 1C).

NF-κB1/RelA is activated at 21% O₂

We then set out to characterize the factors that could have a potential role in inducing inflammation at 21% O₂. To accomplish this, we first identified the transcription factor binding motifs that were overrepresented at the promoters of genes that were differentially expressed at 21% O₂ compared to 10% O₂ using PScan analysis (Zambelli et al., 2009). We found NF-κB1 and RelA to be the top transcription factor binding motifs enriched at the upregulated gene promoters (Fig. 3A). NF-κB1 and RelA are members of the NF-κB family transcription factors, which are major regulators of inflammatory response (Hayden and Ghosh, 2008). In contrast, we did not find enrichment of NF-κB1 or RelA binding motifs at the promoters of genes that were downregulated at 21% O₂ (Fig. 3B).

Next, we identified the upstream transcription factor regulatory network that is likely responsible for the observed gene expression profiles in 21% O₂ cultured cells, using upstream regulator analysis (URA) (Kramer et al., 2014). URA predicted pro-inflammatory cytokine TNF as the top upstream regulator at 21% O₂ (Fig. 3C). In addition, inflammation associated cytokines (IL1B, IFNG) and transcription factors (NFκB, STAT3) were also among the top upstream regulators predicted by URA (Fig. 3C). These results suggest potential activation of inflammatory response cascade at 21% O₂. Consistent with the transcription factor binding motif analysis (Fig. 3A), the URA causal network analysis predicted significant activation of NF-κB complex (Fig. 3D), a major downstream effector of activated TNF (Micheau and Tschopp, 2003). Interestingly, we found several NF-κB family members including *NF-κB1* (*p50/p105*), *NF-κB2* (*p52/p100*), *RelA/p65* and *RelB* to be transcriptionally upregulated at 21% O₂, compared to 10% O₂ (Fig. 3E). Furthermore, several NF-κB target genes including *CXCL2*, *CXCL5*, *CSF1* and *TNFRSF9* were also upregulated at 21% O₂ (Fig. 3F). In summary, enrichment of NF-κB1/RelA binding motifs

at the upregulated gene promoters (Fig. 3A), significant activation of NF- κ B transcriptional network (Fig. 3D) as well as upregulation of NF- κ B family genes (Fig. 3E) and NF- κ B target genes (Fig. 3F) collectively suggest NF- κ B as a major mediator of the cell's response to 21% O₂. To establish a functional role for NF- κ B1/RelA in activating pro-inflammatory genes at 21% O₂, we next asked if there is a differential NF- κ B1/RelA binding at their promoters in response to varying O₂ levels. To accomplish this, we performed chromatin immunoprecipitation (ChIP) analysis using antibodies against RelA and evaluated its binding to several known NF- κ B target genes that were upregulated at 21% O₂ and contained NF- κ B binding motifs at their promoters (PScan analysis). We found significantly increased RelA binding at 21% O₂, compared to 10% O₂ (Fig. 3G), suggesting NF- κ B dependent transcriptional activation of these genes.

Sustained elevation of oxidative stress and inflammation occurs at 21% O₂

We next asked if the increased oxidative stress and inflammation observed in cells grown at 21% O₂ for 72 h, is a transient, short-term phenomenon or a long-term effect on the cells. To accomplish this, we cultured BEAS-2B cells for 3 weeks at 21% and 10% O₂. Similar to the effect of 21% O₂ on short-term (72 h) cultured cells, the long-term (3 weeks) cultured cells showed higher ROS levels (Fig. 4A) and antioxidant depletion (Fig. 4B), suggesting sustained oxidative stress. In addition, GO analysis showed that the genes upregulated at 21% O₂ were associated with inflammatory response (Fig. 4C) and TNF and NF- κ B signaling pathways (Fig. 4D). In contrast, the genes downregulated at 21% O₂ were associated with biological processes including cell proliferation and migration (Fig. 4E) and pathways including extracellular matrix organization and focal adhesion (Fig. 4F). These results suggest the cell's inability to acclimatize to chronic oxidative stress. Consistent with the pathway analysis (Fig. 4D), the NF- κ B family genes (Fig. 4G) and several NF- κ B target genes (Fig. 4H) were also upregulated at 21% O₂. Moreover, URA causal network analysis predicted TNF as the top activated upstream regulator (Fig. 4I), potentially responsible for the observed gene expression profile. NF- κ B complex was also predicted to be activated (Fig. 4I), indicating chronic inflammatory response at 21% O₂. These results suggest that the stress induced by higher O₂ levels is not merely a transient effect, but a sustained one that continues to be present and not attenuated after 3 weeks of cell culture.

RelA knockdown downregulates pro- inflammatory cytokines at 21% O₂

Our results suggest NF- κ B1/RelA complex as an important mediator of inflammatory response in cells grown at 21% O₂. Therefore, we reasoned that depletion of RelA would decrease the inflammatory response in cells cultured at 21% O₂. To test this, we knocked down RelA in BEAS-2B cells using siRNAs. Evaluation of the mRNA (Fig. 5A) and protein levels (Fig. 5B) 72 h post siRNA transfection showed significant reduction in the levels of RelA. Following this, the RelA knockdown (KD) cells were cultured at 10% or 21% O₂ for 72 h. Examination of RelA mRNA and protein levels confirmed the maintenance of RelA depletion 6 days post transfection (Fig. 5C and 5D).

To investigate whether RelA depletion affected the inflammatory response, we examined the mRNA levels of several pro-inflammatory cytokines that were upregulated at 21% O₂, compared to 10% O₂ in the wild-type (WT) cells (Fig. 3F). As expected, RelA KD resulted

in significant downregulation of several pro-inflammatory cytokine genes at 21% O₂ (Fig. 5E). Interestingly, the expression levels of several genes in RelA KD cells at 21% O₂ were similar to the levels observed in WT cells at 10% O₂ (Fig. 5E), further implicating NF-κB dependent processes in the increased inflammatory response observed at 21% O₂. Taken together, these results strongly suggest NF-κB1/RelA as a major transactivator of inflammatory response at 21% O₂.

Cells cultured at 10% O₂ exhibit lower oxidative stress upon H₂O₂ exposure

In order to gain insight into the response of cells grown under different O₂ levels to externally induced stress, we treated BEAS-2B cells cultured at 21% and 10% O₂ to 150 μM H₂O₂ for 72 h. We observed higher ROS levels (Fig. 6A) and lower anti-oxidant levels (Fig. 6B) in H₂O₂ exposed cells cultured at 21% O₂, compared to those cultured at 10% O₂. Moreover, the rate of cell proliferation was lower at 21% O₂ (Fig. 6C). These results suggest that the cells grown at 21% O₂ experience elevated oxidative stress compared to the cells grown at 10% O₂. In addition, biological functions including apoptosis, response to wounding and inflammatory response were significantly elevated at 21% O₂ (Fig. 6D). In contrast, no significant apoptosis or inflammatory response was observed in 10% O₂ grown cells (Fig. 6E). Taken together, these results suggest that the cells cultured under lower oxygen levels display higher ability to tolerate stress when exposed to oxidative stress causing agents.

Discussion

In vivo, the cells are exposed to O₂ levels ranging from 2–9% depending on the tissue (Brahimi-Horn and Pouyssegur, 2007). For example, while brain cells normally experience 4.4% O₂ (Dings et al., 1998; Schultheiss et al., 1987), the kidney cells are exposed to 9.5% O₂ (Muller et al., 1998). However, cell culture studies are almost exclusively carried out at ambient atmospheric O₂ levels (21%). Currently, non-standard, low O₂ (1–5%) culture conditions are limited to primary cells (Carswell et al., 2000; Estrada et al., 2012; Fehrer et al., 2007; Garreta et al., 2014; Guo et al., 2013; Saito et al., 1995). Several studies have reported improved cell growth and life span, in addition to reduced chromosomal abnormalities in primary cells cultured at lower O₂ levels (Estrada et al., 2012; Fehrer et al., 2007; Ho et al., 2007). Although accumulating evidence suggest increase in oxidative stress and loss of cell viability at 21% O₂ (Alaluf et al., 2000; Balin et al., 2002; Boregowda et al., 2012; Busuttil et al., 2003; Chakravarthi et al., 2006; Halliwell, 2003; von Zglinicki et al., 1995), the cellular pathways affected are not fully understood.

To understand the mechanisms that underlie the apparent adverse effects of 21% O₂ on cultured cells, it is important to identify the genes and pathways that are affected at higher O₂ levels. Moreover, since culturing cells at 21% O₂ is traditionally the most preferred cell culture method due to ease-of-use, it is important to understand the molecular pathways and biological processes that could potentially be affected at this non-physiological, higher O₂ level. In this study, by examining gene expression changes and identifying the pathways altered at 21% O₂, we report inflammation mediated by NF-κB signaling as a major consequence of culturing cells at 21% O₂.

The human bronchial epithelial BEAS-2B cells are immortalized, non-cancerous cells, normally cultured at 21% O₂. However, under physiological conditions the lung cells are exposed to 6–14% O₂ (Le et al., 2006; Wild et al., 2005). Therefore, we decided to compare the effects of physiological and higher O₂ levels on BEAS-2B cells by culturing them at 10% and 21% O₂, respectively. Analysis of the differentially expressed genes revealed inflammation as a predominant outcome of culturing cells at 21% O₂ (Fig. 2). Interestingly, we found transcriptional activation of several genes involved in regulation of immune response and cytokine production at 21% O₂. Furthermore, we found oxidative stress and inflammation induced by 21% O₂ to be a sustained, long-term effect that did not exhibit adaptation even after 3 weeks (Fig. 4).

To gain insight into the activation of the pro-inflammatory genes, we compared the phenotypic characteristics of the cells grown at 21% and 10% O₂ and also performed detailed pathway analysis of the differentially expressed genes. The cells grown at 21% O₂ showed increased ROS production and decreased glutathione levels, compared to the 10% O₂ cultures, indicating disruption of redox balance. Moreover, we found increase in unfolded protein response at 21% O₂ (Fig. 2A), which further indicated endoplasmic reticulum (ER) stress and utilization of glutathione (Chakravarthi et al., 2006). Previous studies have shown that ER stress and imbalance in cellular redox status could induce the pro-inflammatory TNF network (Galter et al., 1994; Haddad et al., 2000; Hu et al., 2006). Interestingly, we detected TNF as one of the major upstream regulators activated at 21% O₂ (Fig. 3C and D). TNF- α signaling mediates a multitude of inflammatory events in the lungs including activation of NF- κ B signaling (Micheau and Tschopp, 2003). Our causal network analysis revealed NF- κ B complex as the major downstream effector activated by TNF (Fig. 3D). Besides TNF, it is likely that increased ROS levels, as well as altered cellular redox equilibrium, particularly intracellular thiol status could also be involved in the activation of NF- κ B complex (Galter et al., 1994; Gloire et al., 2006; Haddad et al., 2000).

NF- κ B activation plays an essential and evolutionarily conserved role in coordinating immune and inflammatory responses to various stress signals (Hayden and Ghosh, 2008). Stress inducible expression of a large number of genes involved in inflammatory response, immunity and apoptotic processes are NF- κ B dependent (Hayden and Ghosh, 2008). Interestingly, our results reveal NF- κ B1 and RelA binding motifs as the top enriched motifs at the promoters of genes upregulated at 21% O₂. In addition, we found significant increase in RelA binding at several pro-inflammatory cytokine gene promoters upregulated at 21% O₂ (Fig. 3G), implicating NF- κ B in the regulation of transcriptional program at higher O₂ levels. Moreover, RelA knockdown resulted in downregulation of several pro-inflammatory cytokines at 21% O₂ (Fig. 5E). These results point to NF- κ B1/RelA being a key mediator of the inflammatory response at 21% O₂. Taken together, our studies suggest that culturing BEAS-2B cells at 21% O₂ leads to increased ROS and decreased antioxidant levels, which in turn activates the TNF network. Activated TNF induces NF- κ B1/RelA transcriptional regulators and the downstream pro-inflammatory NF- κ B1/RelA target genes (Fig. 7).

Unlike primary cells, the immortalized cells are programmed to evade normal cellular senescence and continue to proliferate even under the stress caused by exposure to 21% O₂ (Shay et al., 1993; Shay and Wright, 2005; Shay et al., 1991). Nevertheless, our results show

that the cells undergo significant and sustained stress when cultured at 21% O₂. Moreover, the cells cultured at 21% O₂ exhibit heightened apoptosis and inflammatory response when exposed to an external stress (H₂O₂) compared to the cells grown at 10% O₂ (Fig. 6). In conclusion, although culturing immortalized cells at 21% O₂ is a standard practice, the stress to which the cell is already subjected to, by being cultured at higher O₂ levels could potentially influence the outcome of the experiments that are conducted with cells cultured in 21% O₂. Therefore, caution should be exercised while interpreting results from cells cultured at 21% O₂. It is plausible that the O₂ sensitivity and the amount of stress that each cell-type undergoes at 21% O₂ may vary depending on the cell-type specific signaling events as well as the tissues from which the cells originated.

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Figure 1. 21% O₂ induces oxidative stress

(A) BEAS-2B cells cultured at 21% O₂ for 72 h showed significantly higher intracellular ROS levels compared to the cells grown at 10% O₂, as measured by the percentage of H₂DCFDA stained cells. (B) The cells cultured at 21% O₂ for 72 h showed lower reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio, suggesting antioxidant depletion. (C) MTT assay showing reduction in cell proliferation rate in cells cultured at 21% O₂. The mean optical density of 0 h time point was normalized to 1. All error bars represent standard deviations for at least two biological replicates. Statistical significance was evaluated using t-test (p<0.05 (*); p<0.005 (**); p<0.0005 (***)).

Figure 2. Pro-inflammatory pathways are upregulated at 21% O₂

(A and B) Functional enrichment analysis showing the major GO biological processes associated with (A) genes upregulated in cells cultured for 72 h at 21% O₂, compared to the cells cultured at 10% O₂ and (B) genes downregulated in cells cultured for 72 h at 21% O₂, compared to the cells cultured at 10% O₂. (C and D) Functional enrichment analysis showing the major canonical pathways associated with (C) genes upregulated in cells cultured for 72 h at 21% O₂, compared to the cells cultured at 10% O₂ and (D) genes downregulated in cells cultured for 72 h at 21% O₂, compared to the cells cultured at 10% O₂. In the panels A and B, circle color indicates relative significance of the GO terms (log p-value) and circle size corresponds to the number of genes annotated to the GO term in the reference database (log size). Gene expression data represent combined analysis of two biological replicates.

Figure 3. Exposure to 21% O₂ causes NF-κB activation

(A) Overrepresented transcription factor binding motifs at the promoters of genes upregulated in cells cultured at 21% O₂, compared to cells cultured at 10% O₂. (B) Overrepresented transcription factor binding motifs at the promoters of genes downregulated in cells cultured at 21% O₂, compared to cells cultured at 10% O₂. (C) Top upstream regulators activated at 21% O₂. (D) TNF-driven mechanistic network showing predicted activation/inhibition status of the connected regulators. Prediction legend, z-score and p-value are provided as inset. (E and F) Gene expression analysis (qPCR) showing upregulation of (E) NF-κB family genes and (F) NF-κB target genes in cells cultured for 72 h at 21% O₂, compared to cells cultured at 10% O₂. mRNA levels of 10% O₂ grown cells were normalized to 1. *GAPDH* was used as internal control. (G) ChIP-qPCR analysis showing enrichment of RelA binding at the promoters of NF-κB1/RelA target genes in the cells grown at 21% O₂. *CDC1*, which does not possess NF-κB1/RelA binding motif, was used as negative control. Fold enrichments of RelA over IgG were calculated and the RelA levels of 10% O₂ cells were normalized to 1. All error bars represent standard deviations for at least two biological replicates. Statistical significance was evaluated using t-test (p<0.05 (*); p<0.005 (**); p<0.0005 (***)).

Figure 4. Sustained elevation of oxidative stress and inflammation occurs at 21% O₂
 (A) BEAS-2B cells cultured at 21% O₂ for 3 weeks showed significantly higher intracellular ROS levels compared to the cells grown at 10% O₂. (B) Cells cultured at 21% O₂ for 3 weeks show decrease in antioxidant levels. (C and D) Functional enrichment analysis showing the major (C) GO biological processes and (D) canonical pathways associated with the genes upregulated in BEAS-2B cells cultured for 3 weeks at 21% O₂, compared to the cells cultured at 10% O₂. (E and F) Functional enrichment analysis showing the major (E) GO biological processes and (F) canonical pathways associated with the genes downregulated in BEAS-2B cells cultured for 3 weeks at 21% O₂, compared to the cells cultured at 10% O₂. In the panels C and F, circle color indicates relative significance of the GO terms (log p-value) and circle size corresponds to the number of genes annotated to the GO term in the reference database (log size). (G and H) Gene expression analysis (qPCR) showing upregulation of (G) NF-κB family genes and (H) NF-κB target genes in cells cultured at 21% O₂ for 3 weeks, compared to 10% O₂ cultured cells. mRNA levels of 10% O₂ grown cells were normalized to 1. *GAPDH* was used as internal control. (I) TNF-driven mechanistic network showing the predicted activation/inhibition status of the connected regulators. Prediction legend, z-score and p-value are provided as inset. Gene expression data represent combined analysis of two biological replicates in each condition. All error bars represent standard deviations for at least two biological replicates. Statistical significance was evaluated using t-test (p<0.05 (*); p<0.005 (**); p<0.0005 (***)).

Figure 5. RelA knockdown downregulates pro-inflammatory cytokine gene expression at 21% O₂

(A and B) Significant decrease in RelA (A) mRNA levels (qPCR) and (B) protein levels (western blotting) occurred in BEAS-2B cells transfected with RelA siRNAs for 72 h. (C and D) The decrease in RelA (C) mRNA and (D) protein levels were maintained in BEAS-2B cells 6 days post RelA siRNA transfection in both 10% and 21% O₂. For qPCR *GAPDH* was used as internal control. For western blotting analysis β-actin was used as loading control. (E) qPCR analysis showing downregulation of several inflammatory cytokines in RelA knockdown cells. *GAPDH* was used as internal control. All error bars represent standard deviations for at least two biological replicates. Statistical significance was evaluated using t-test (p<0.05 (*); p<0.005 (**); p<0.0005 (***); NS: Not significant).

Figure 6. 10% O₂ cultured cells undergo less stress upon H₂O₂ exposure

BEAS-2B cells cultured at 21% or 10% O₂ were treated with 150 μ M H₂O₂ for 72 h. (A) H₂O₂ exposed cells show elevated ROS levels at 21% O₂. (B) Reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio was lower in cells cultured at 21% O₂, suggesting antioxidant depletion. (C) MTT assay showing reduction in cell proliferation at 21% O₂. The mean optical density of 0 h time point was normalized to 1. All error bars represent standard deviations for at least two biological replicates. Statistical significance was evaluated using t-test ($p < 0.0005$ (***); $p < 0.00005$ (****)). (D) GO biological processes associated with the genes upregulated at 21% O₂ in H₂O₂ exposed cells. (E) GO biological processes associated with the genes upregulated at 10% O₂ in H₂O₂ exposed cells. In the panels D and E, circle color indicates relative significance of the GO terms (log p-value) and circle size corresponds to the number of genes annotated to the GO term in the reference database (log size). Gene expression data represent combined analysis of two biological replicates.

Figure 7. Proposed model for oxidative stress induced inflammatory response in BEAS-2B cells cultured at 21% O₂

Exposure to 21% O₂ results in increased ROS and decreased antioxidant levels indicating oxidative stress. The elevated oxidative stress activates TNF pathway. Activated TNF induces activation of NF-κB family transcriptional regulators. Increase in NF-κB1/RelA complex binding to its target pro-inflammatory genes result in their upregulation. Green arrows indicate the direction of activation and black arrow indicates NF-κB1/RelA translocation to the nucleus.