Differential Effects of Hypoxic Stress in Alveolar Epithelial Cells and Microvascular Endothelial Cells

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Abstract
Under hypoxic conditions eukaryotic cells and tissues undergo adaptive responses involving glycolysis, angiogenesis, vasoconstriction and inflammation. The underlying molecular mechanisms are not yet fully elucidated and are most likely cell and tissue specific. In the lung, alveolar epithelial cells and microvascular endothelial cells are highly sensitive to hypoxia and together orchestrate a rapid and sustained adaptive response. We examined the effect of different oxygen tensions on cell viability, glucose metabolism, key transcription factors and signaling molecules, in alveolar epithelial cells (A549) and microvascular endothelial cells (HMEC-1). Both cell types tolerated hypoxia without detectable cell injury. Hypoxia induced glycolysis in both epithelial and microvascular endothelial cells, although A549 cells exhibited a higher rate of glucose consumption. The transcription factor CREB (cAMP response element binding protein) was activated with decreasing oxygen tensions in both cell types. This effect was again more marked in A549 cells, demonstrating epithelial cells to be more oxygen sensitive. Activating Transcription Factor 3 (ATF-3) was heavily induced by hypoxia in A549 cells but not in HMEC-1 cells. Both cell types exhibited hypoxia induced secretion of VEGF and IL-6. Secretion of the vasoconstrictor endothelin-1 (ET1) was increased by hypoxia in HMEC-1 cells but decreased in A549 cells. These data reveal that both cell types exhibit an adaptive response to hypoxia but alveolar epithelial cells are generally more sensitive. ET-1 was oppositely regulated by decreased oxygen tensions in the investigated cell types. The present study further elucidates the adaptive molecular mechanisms in pulmonary hypoxia and demonstrates cell specific responses.

Introduction
Hypoxia occurs in cells and tissues when the demand for oxygen, to maintain normal cellular function, outstrips supply [1-4]. Cells adapt to hypoxic stress in a multitude
of ways, including; induction of anaerobic metabolism, increasing haemocrit levels and increasing vascularisation [5-8]. Without such adaptation, cell death would occur due to insufficient ATP supply. Within the lung, hypoxia plays a role in disease progression in conditions such as pulmonary hypertension, cystic fibrosis and chronic obstructive pulmonary diseases (COPD) [1, 5, 9-12]. Important adaptive mechanisms involving alterations in gene expression are necessary to maintain lung functions.

The underlying transcriptional mechanisms responsible for altered gene expression are continuing to be unraveled. It has been established that hypoxia inducible factor 1 (HIF-1), a primary driver of hypoxic gene expression in many cells and tissues [1, 13-21], plays a major role in lung adaptation to hypoxia [22-25]. Recent studies have suggested the role of other transcription factors for hypoxia induced lung adaptation, including the ATF/CREB family [26]. CREB (cAMP response element binding protein) and genes that contain CREB family binding sites, such as Activating Transcription Factor 3 (ATF-3), Endothelin 1 (ET-1) and Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) have been found to be activated during hypoxia in the in vivo mouse lung [26]. Indeed, CREB has been found to regulate the transcription of a large number of genes involved in glucose homeostasis, inflammation, cell survival and signal transduction [21, 27-33]. It has become increasingly more appreciated that the molecular mechanisms governing lung adaptation to hypoxia are cell type specific and strictly related to the functional interaction among neighboring cells. The close association between alveolar epithelial and microvascular endothelial cells is essential for guaranteeing the efficient gas exchange between the blood and the alveolar space. Hypoxia induced alterations in gene expression in either cell type has major consequences for the maintenance of normal tissue function.

It was the aim of this study to examine differential hypoxic responses in pulmonary epithelial and microvascular endothelial cells, so that we may better understand their individual contribution to lung adaptation in hypoxia related disease states. Here we show that although both cell types were responsive to altered oxygen tensions, alveolar epithelial cells were generally more sensitive than microvascular endothelial cells. Additionally we observed an opposing effect of hypoxia on ET-1 secretion in both cell types studied.

Materials and Methods

Chemicals
All chemicals were purchased from Sigma (Vienna, Austria) unless otherwise mentioned.

Cell culture
A549 cells (human lung adenocarcinoma epithelial cells) were purchased from SkinEthic Laboratories (Nice, France). Cells were cultured in 10 cm culture dishes and maintained at 37 °C in a humidified atmosphere containing 95% air and 5% CO₂. The medium used for propagation was DMEM with 5 mM glucose supplemented with 7% fetal calf serum. HMEC-1 cells (human dermal microvascular endothelial cells), originally described by Ades et al. [34], were maintained in MCDB-131 medium, supplemented with 7% fetal calf serum (FCS; Biochrom, Berlin, Germany), 2 mM glutamax, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 μg/ml hydrocortisone and 10 ng/ml EGF. Both cell types were fed three times weekly and subcultivated by trypsinization when nearly confluent.

Experimental design
Hypoxia chambers were constituted of an air tight plastic lunch box (Curver, Luxembourg) with two holes drilled in the lid. A one way valve was placed in each hole, one allowing gas in and one allowing gas out (Fig 1.). Hypoxia chambers were humidified and pre-warmed to 37 °C for experimentation. A549 and HMEC-1 cells were trypsinized and seeded into either 6-well plates or on glass coverslips in 12 well plates for immunofluorescence. When cells reached full confluence, monolayers were placed into the hypoxia chambers (Fig 1.). Gassing was commenced with pre-warmed humidified N₂, containing 5% CO₂ and 15%, 7%, 1% and 0% O₂. Gas mixes were purchased from Linde Gas GmbH, Austria. For each oxygen concentration, one hypoxia chamber was used. Oxygen concentrations within the hypoxia chambers were checked intermittently during the experiments using a flow through optical oxygen sensor (Fibox 3, Presens, Regensburg, Germany) measuring the out-flowing air. Experiments were routinely conducted for 24 h.

Glycolysis
Glucose was measured in supernatant medium using a colorimetric assay as originally described by Caroll [35] and optimized by Jennings [36]. The assay is based on the conversion of glucose to glucose-6-phosphate (G6P) by hexokinase. Lactate was measured in the supernatant medium using a colorimetric assay as originally described by Babson [37] and optimized by Jennings [36]. The lactate assay is based on the conversion of lactate to pyruvate by lactate dehydrogenase (LDH).

Cytotoxicity
Release of the cytosolic enzyme lactate dehydrogenase (LDH) into the supernatant medium was assayed using a commercially available assay (Roche, Mannheim, Germany).
Quantitative real-time PCR

Cells were exposed to 15%, 7%, and 1% oxygen. RNA was extracted with an RNA high pure isolation kit (11 828 665 001 Roche Diagnostics, Applied Science, Vienna, Austria). RNA was quantified by the Ribogreen assay (R11490, Invitrogen, Lofer, Austria) according to the manufacturer’s instructions. RNA was transcribed to cDNA with *Escherichia coli* reverse transcriptase, oligo (dt), and random primers (Qiagen, Austria). Commercially available predesigned primers for ATF-3 (Applied Biosystems, Austria) were used for determination of gene expression levels. 18S RNA (Applied Biosystems) was used as the house keeping gene (endogenous control). PCR reagent mixes contained 900 nM sense and antisense primer, 200 nM probe and a TaqMan Mix (Applied Biosystems). Thermal cycling profile started with 2 min at 50°C (RNase inhibitor activation) and 10 min at 95°C to activate polymerase. Cycles of 95°C for 15 s, followed by 60°C for 1 min were repeated 40 times. Samples were run in duplicate, and the gene expression levels were calculated with the comparative threshold cycle (Ct) method (2^-ΔΔCt method). For each sample, the ATF-3 expression was determined as relative amount with respect 18S expression level and converted to fold over control (normoxia, 15% O₂) values. Data were acquired with the ABI PRISM 7700 and evaluated with the SDS 1.9.1 software package.

Signaling molecule release

Supernatants were collected and ET-1, VEGF and IL-6 were measured using an enzyme immunoassay (DuoSet, R & D Systems, Minneapolis, MN), according to the manufacturer’s instructions. Amplex Red Ultra (20 μM) (Molecular Probes) and 5 mM H₂O₂ dissolved in PBS was used for horseradish peroxidase (HRP) detection. Fluorescence (excitation wavelength: 540 nm; emission wavelength: 595 nm) was detected with a microtiter plate reader TECAN GENios Plus from TECAN GmbH Swiss. Measured concentrations (pg/ml) were converted to fold over control (15% O₂).

Phospho-CREB (ser133) detection

Activation of CREB was detected using a phospho-specific antibody directed towards serine 133. Cells were exposed to 15%, 7% and 1% oxygen. Nuclear protein lysates were prepared as described previously [26]. Phospho-CREB (ser133) was detected in the nuclear extracts using an enzyme immunoassay (DuoSet, R & D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Immunofluorescence

After exposure to 15% and 1% oxygen, cell monolayers were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Monolayers were washed 5 times in PBS followed...
by a 30 min incubation in blocking solution (5% BSA, 1% Triton-X-100 in PBS). Cells were incubated with primary antibody (1:200: mouse monoclonal ATF-3 antibody, ab 58668, Abcam; 1:800 HIF-1 alpha polyclonal antibody, #3716, Cell Signaling; 1:200 rabbit CREB-1 polyclonal antibody, sc-186, Santa Cruz Biotechnology; 1:200 p-CREB-1 (Ser 133) rabbit polyclonal antibody, sc-101663, Santa Cruz Biotechnology) in 0.5% BSA, 0.1% Triton-X 100 in PBS over night at 4°C. After washing, secondary antibody (1:800 Alexa Fluor 488 rabbit anti-mouse IgG, A11059, Molecular Probes and 1:800 Alexa Fluor 488 donkey anti-rabbit IgG, A21206, Molecular Probes) was applied for 1 hour at room temperature. Coverslips were washed and mounted into slides with 3 mg/μl p-Phenylen-Diamine glycerol. Fluorescent images were obtained with a Zeiss Axiophot fluorescent microscope using a 63 X 1.40 numerical aperture oil immersion lens and acquired using META VUE image processing software (Universal Imaging, Downingtown, PA).

**Results**

**Effect of oxygen tension on plasma membrane integrity**

Lactate dehydrogenase (LDH) released into the supernatant is a parameter of plasma membrane integrity [38]. In order to investigate hypoxia (7% and 1% O₂) and anoxia (0% O₂) induced cell injury, LDH activity was measured in cell culture supernatant (Fig. 2). Hypoxia did not cause plasma membrane damage neither in A549 nor in HMEC-1 cells. Anoxia however, caused cell injury in A549 (2.62-fold over control ± 0.42) and a slight but still significant (1.2-fold over control ± 0.09) cell injury in HMEC-1 cells.

**Effect of oxygen tension on glucose metabolism**

Eukaryotic cells quickly respond to hypoxia insults by a shift from aerobic to anaerobic metabolism. Therefore, we measured the levels of glucose and lactate in the supernatants of our cell models incubated for 24 hours with 15%, 7%, 1% and 0% O₂ (Fig. 3A and B). After a 24 h incubation under 15% oxygen, glucose levels were reduced from 5 mM to 1.82 ± 1.26 mM (mean ± SD) in A549 cells and from 5 mM to 4.27 ± 0.57 mM in A549 cells and from 5 mM to 4.27 ± 0.57 mM in...
HMEC-1 cells. Under these conditions lactate levels were increased from 0.2 mM to 4.69 ± 1.49 mM in A549 cells and from 0.2 mM to 2.32 ± 0.65 mM in HMEC-1 cells. Only in A549 cells glycolysis was induced by 7% O\(_2\) exposure; under this condition a 82% glucose reduction (as compared to the control) and a 53% increase of lactate production was observed. Glycolysis was induced in both cell types at 1% and 0% oxygen. A549 cells exhibited a 96% and 95% supernatant glucose reduction at 1% and 0% O\(_2\) respectively. HMEC-1 demonstrated a 13% and 17.5% supernatant glucose reduction at 1% and 0% O\(_2\) respectively. This hypoxia/anoxia induced glucose consumption was paralleled by a significant increase in lactate production (P < 0.001) in both cell types.

**Effect of oxygen tensions on transcription factor activation and expression**

We performed immunocytochemistry using antibodies directed against HIF-1 alpha, ATF-3, CREB and phospho-CREB to determine activation of these transcription factors in hypoxia. Immunofluorescence analyses (Fig. 4) demonstrated a strong hypoxia (1% O\(_2\)) induction of HIF-1 alpha, ATF-3 and phospho-CREB in epithelial cells. In microvascular endothelial cells exposed to 1% oxygen, HIF-1 alpha appears to be more localized in the nuclei compared to the control conditions (15% O\(_2\)). No significant change in ATF-3 and phospho-CREB levels was observed in microvascular endothelial cells. Hypoxia treatment did not influence total CREB levels in both cell types.
Real time PCR results revealed a 3.06-fold ± 0.0038 (fold over control ± SD) ATF-3 induction in epithelial cells exposed to 7% oxygen and a 117-fold ± 26.35 induction in the same cell type exposed to 1% oxygen (Fig. 5 A). ATF-3 was unchanged by hypoxic exposure in microvascular endothelial cells.

Phosphorylated CREB measured by EIA in nuclear extracts was slightly increased (1.19 ± 0.13, P= 0.0338) in A549 cells at 7% and 2.73-fold ± 0.62 increased at 1% (Fig. 5 B). In microvascular endothelial cells no significant change in phospho-CREB was observed at 7% oxygen whereas, it was induced 1.58-fold ± 0.28 at 1% oxygen.

Effect of oxygen tension on the secretion of signaling molecules

Given the importance of vasoconstriction, immune regulation and angiogenesis during hypoxia, we examined the effect of oxygen reduction and deprivation on the secretion of specific hypoxia responsive proteins: endothelin-1 (ET-1), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) (Fig. 6 and 7). In A549 cells at 7% a slight decrease of ET-1 secretion (0.92 ± 0.02) was observed. This decrease was more marked at 1% (0.53-fold ± 0.005) and at 0% (0.52-fold ± 0.008). Conversely, in HMEC-1 cells both anoxia and hypoxia resulted in a increase of ET-1 secretion (to 1.1 fold ± 0.009 at 7%, to 1.53 fold ± 0.02 at 1% and to 1.38 fold ± 0.01 at 0% oxygen tension). Incubation with 7% oxygen resulted in a 2.08-fold ± 0.13 increase in VEGF secretion in epithelial cells, whereas no significant difference was observed in microvascular endothelial cells exposed at the same oxygen tension. At 1% and 0%,
VEGF secretion was strongly induced in both A549 (1%: 2.49-fold ± 0.1; 0%: 2.62-fold ± 0.09) and HMEC-1 (1%: 2.04-fold ± 0.07; 0%: 3.25 ± 0.12) cells. No significant change in IL-6 release was observed at 7% oxygen in either cell type. In epithelial cells IL-6 was induced 1.56-fold ± 0.16 at 1% and 1.64-fold ± 0.14 at 0%; in microvascular endothelial cells IL-6 was induced 1.49-fold ± 0.14 at 1% and 1.35-fold ± 0.14 at 0%.

Discussion

Altering environmental oxygen tension result in physiological and/or pathophysiological cellular and tissue adaptive responses, associated with differential gene expression. A complex and interactive feedback relationship exists among the products of genes regulated by hypoxia. These mechanisms are likely to be cell and tissue specific and related to cell function. In the lung, epithelial and microvascular endothelial cells are highly sensitive to hypoxia and together orchestrate a rapid and sustained adaptive response. The aim of this study was to compare the effects of hypoxia on epithelial and microvascular endothelial cells. We therefore exposed A549 and HMEC-1 cells for 24 hours to different oxygen tensions and examined cellular viability, glycolysis, transcription factor activation, gene expression and signaling pathways.

A major factor in cell survival under low oxygen conditions is the ability of cells to increase glycolysis in order to maintain sufficient ATP levels [7, 39]. Anoxia induced mild cell injury in both epithelial and microvascular endothelial cells, while 1 and 7% oxygen was tolerated by both cell types. Glycolysis was dose dependently increased in both HMEC-1 and A549 cells due to decreased oxygen tensions; however epithelial cells were more sensitive and more inducible. These altered oxygen sensitivities in the two cell types may be related to different basal glycolysis rates, as under control conditions A549 cells consumed more glucose and produced more lactate than HMEC-1 cells.

HIF-1 alpha, CREB and ATF3 have been shown to play a role in hypoxia related pulmonary diseases [13, 23, 24, 26, 40-42]. Using immunofluorescence we could demonstrate a strong increase of nuclear HIF-1 alpha, phospho CREB and ATF-3 in A549 cells, whereas HMEC-1 cells exhibited a slight induction of HIF-1 alpha and CREB. No change of ATF-3 was observed in either normoxia or hypoxia in microvascular endothelial cells. The qualitative analysis was confirmed by the ATF-3 mRNA expression analyses and by the quantitative measurement of activated phospho-CREB in nuclear protein extracts of both cell types. These results are consistent with the increase in glycolysis in A549 cells indicating an increased sensitivity to hypoxia. Moreover, all the three transcription factors are involved in glucose metabolism. For example HIF-1 alpha is known to induce the expression of several genes regulating glycolysis such as hexokinase-2, phosphofructokinase, GAPDH, enolase-1 and LDHA [13]. Approximately
25% of the genes regulated by CREB play a role in the control of metabolic processes [26] and it has been observed that inhibition of CREB induces fasting-hyperglycemia and reduces expression of gluconeogenic genes such as phosphoenolpyruvate carboxykinase and glucose-6- phosphatase [43]. Interestingly, it has also been demonstrated that ATF-3 is strongly induced in renal epithelial cells as a result of medium exhaustion [36] suggesting that selective activation of ATF-3 in A459 cells is a consequence of hypoxia induced glucose deprivation. Our findings therefore, suggest a strong relationship between the activation of HIF-1 alpha, CREB and ATF-3 and the observed glycolytic response in both cell types.

ET-1 is the most potent vasoconstrictor released by endothelial cells and functions as a major paracrine regulator of vascular tone. The vascular response has multiple components including acute hypoxic-induced regional pulmonary vasoconstriction which may lead to a rapid optimization of tissue oxygenation and improvement of perfusion-ventilation ratio [44]. Our results show that ET-1 release is induced by hypoxia and anoxy from microvascular endothelial cells. However, ET-1 secretion was suppressed by hypoxia and anoxy in pulmonary epithelial cells. This phenomenon has been reported previously for both A549 cells and primary rat alveolar type II cells [45]. Although animal studies indicate that ET-1 can exert profound effects on the function of alveolar epithelial cells, the mechanisms of ET-1 processing within alveolar epithelial cells are not known at this time. In rat alveolar type II cells ET-1 caused concentration-dependent secretion of the surfactant phosphatidylcholine suggesting a role for ET-1 secreted by endothelial cells in the regulation of surfactant proteins [46]. The observed reduction in ET-1 secretion from A549 is consistent with the fact that production of the surfactant protein C is decreased in A549 under hypoxic conditions [47]. Furthermore alveolar epithelial cells express high levels of endothelin receptors, [48] and its production is modulated by inflammatory mediators. As ATF3 was observed as the only transcription factor examined to be entirely selectively activated in epithelial cells it is tempting to speculate that ATF-3 might be responsible for down regulation of ET-1 as the effect of ATF-3 on ET-1 has not previously been studied.

In the lung, VEGF functions as a mitogen for endothelial cells, mediating endothelial cell growth and tissue neovascularization [49]. Hypoxia induced VEGF secretion from epithelial cells at 7% oxygen, whereas there was no change in VEGF secretion from endothelial cells between the control and the 7% oxygen exposure. The trend of VEGF secretion in response to hypoxia was very similar to the observed lactate production in the supernatant. This may be explained by the fact that VEGF is activated through the transcriptional regulation of HIF-1 alpha (which binds to the HRE region of the VEGF promoter). Several studies investigating the effect of hypoxia on the activation of HIF-1 transcription factor have also demonstrated VEGF induction is paralleled by an increased glycolysis [15, 50]. Furthermore the receptors for VEGF (Flt-1 and KDR/flk ) are upregulated in vascular tissue in parallel with their respective ligands by low oxygen tension [44]. It has been suggested that VEGF secreted from alveolar epithelial cells could target the capillary endothelium and act through VEGF receptors located on the endothelial cells [7].

Hypoxia and anoxy resulted in an increased secretion of the pro-inflammatory cytokine, IL-6 from both cell types. Moreover, IL-6 induction occurred at the same oxygen tensions and was induced to a similar level in both epithelial and microvascular endothelial cells. Several recent studies have reported an active role for IL-6 on pulmonary vascular remodeling and hypoxic pulmonary hypertension in mice [51]. Indeed, recent observations from a microarray study performed on mice exposed to 10% oxygen revealed a cluster of genes upregulated at 24 hrs within the hypoxic lung [26]. This cluster contained many chemokines and cytokines including IL-6 and paralleled an inflammatory response known to occur within the lung on hypoxic exposure [51].

In conclusion, the present study reveals important insights into differential regulation of hypoxic adaptation in lung epithelial and microvascular endothelial cells in the context of cell survival, glucose homeostasis, vascularisation and inflammation. The data presented, increase our understanding of the complex cellular and molecular pathways which govern cell responses in the adaptation to hypoxia within the lung.

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