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Differential Sex-Specific Effects of Oxygen Toxicity in Human Umbilical Vein Endothelial Cells

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Abstract

Despite the well-established sex-specific differences in the incidence of bronchopulmonary dysplasia (BPD), the molecular mechanism(s) behind these are not completely understood. Pulmonary angiogenesis is critical for alveolarization and arrest in vascular development adversely affects lung development. Human neonatal umbilical vein endothelial cells (HUVECs) provide a robust *in vitro* model for the study of endothelial cell physiology and function. Male and Female HUVECs were exposed to room air (21% O₂, 5% CO₂) or hyperoxia (95% O₂, 5% CO₂) for up to 72 hr. Cell viability, proliferation, H₂O₂ production and angiogenesis were analyzed. Sex-specific differences in the expression of VEGFR2 and modulation of NF-kappa B pathway were measured. Male HUVECs have decreased survival, greater oxidative stress and impairment in angiogenesis compared to similarly exposed female cells. There is differential expression of VEGFR2 between male and female HUVECs and greater activation of the NF-kappa B pathway in female HUVECs under hyperoxic conditions. The results indicate that sex differences exist between male and female HUVECs *in vitro* after hyperoxia exposure. Since endothelial dysfunction has a major role in the pathogenesis of BPD, these differences could explain in part the mechanisms behind sex-specific differences in the incidence of this disease.

Keywords

Hyperoxia; Sex; HUVEC; angiogenesis; bronchopulmonary dysplasia

Introduction

Bronchopulmonary dysplasia (BPD) is a debilitating lung disease with long-term consequences and is one of the most common causes for morbidity in premature neonates. It is characterized by arrest in alveolar septation and vascular development. Postnatal exposure to high concentrations of oxygen (hyperoxia) contributes to the development of BPD. Male

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sex is considered an independent predictor for the development of BPD. Despite the well-established sex-specific differences in the incidence of BPD and impaired lung function in males, the underlying molecular mechanism(s) are not completely understood.

Pulmonary angiogenesis is critical for alveolarization, and arrest in vascular development adversely affects lung development [1,2]. The lung is the first organ to be affected following exposure to hyperoxia and all cell types are affected adversely. Among these, the endothelial cells are more sensitive than epithelial cells [3]. Endothelial cell function is crucial for angiogenesis and alveolarization in the neonatal lung. Human umbilical vein endothelial cells (HUVECs) provide a robust *in vitro* model for the study of endothelial cell physiology and pathology [4]. VEGF is a key regulator of angiogenesis, especially in the developing lung. It mediates its actions mainly through two receptor tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (KDR/flk-1). Of these two, VEGFR2 mediates many of the biologic effects like proliferation and migration of endothelial cells [5]. Sex-specific differences in gene expression and function of HUVECs have been described previously [6]. However, sex differences in an *in vitro* model of hyperoxia exposure and the underlying mechanisms, such as differential expression of key angiogenic mediators such as VEGFR2, have not been investigated.

Data from our laboratory and those of other investigators have shown sex-specific modulation of NF- κ B pathway [7–9]. In a model of neonatal hyperoxic lung injury, we showed that there was a greater arrest in lung development (alveolarization and angiogenesis) in male mice. Interestingly, female mice showed increased activation of the NF- κ B pathway in the lungs compared to males. The larger decrease in angiogenesis in males exposed to hyperoxia may have been in part due to decreased NF- κ B activation [9]

We sought to determine whether innate sex differences independent of circulating sex steroids contributed to endothelial cell survival and injury in response to hyperoxia. We hypothesized that male HUVECs exposed to hyperoxia will show greater cell death, oxidative stress and impaired angiogenesis compared to similarly exposed female HUVECs due to differential modulation of key angiogenic pathways.

Methods

Cell culture and hyperoxia treatment

Human umbilical endothelial cells (male and female) were obtained from Lonza (Lot. 0000241352, 0000243641, 0000315132, 0000321138, CC-2517; Lonza, Allendale, NJ) and maintained in EGM-plus medium (CC-5036, Lonza) with SingleQuots (CC-4542, Lonza) at 37°C in 5% CO₂. Male and Female HUVECs were used from passages 3 – 6.

Exposure of cells to hyperoxia

Before hyperoxia treatment, 1×10^5 Male or Female HUVEC cells were seeded in a 6 mm dish. 24 hr later, these cells were incubated at 37°C in room air condition (21% O₂, 5% CO₂) or in hyperoxia (95% O₂, 5% CO₂) as described before [10]. For each protocol, three independent experiments were performed. After hyperoxia exposure, cells and cell culture supernatants were harvested at 0, 8, 24, 48 or 72 hr

Trypan blue staining

Cells were stained with Trypan blue dye (0.04% in phosphate-buffered saline; 1:1) after exposure to room air or hyperoxia. The cell viability was calculated using TC20 Automated Cell Counter (Bio-Rad, Hercules, CA).

CCK-8 Assay

For measuring cell viability we performed the cell proliferation assay (CCK-8) as per the manufacturer (Sigma-Aldrich, St. Louis, MO) recommendations. 100ul of cell suspension containing 5000 cells was seeded in a 96-well plate. After 24 h pre-incubation at 37°C in 5% CO₂, the cells were incubated in room or hyperoxia (95% O₂, 5% CO₂). 10 µl CCK-8 solution was added to each well in the last 1 hour of incubation. Absorbance at 450 nm was measured at 0, 8, 24 and 48 hr after hyperoxia incubation using a microplate reader (M3 SpectraMax, Molecular Devices Corporation, Sunnyvale, CA).

H₂O₂ Assay

The ROS-Glo™ H₂O₂ Assay (Promega Inc., Madison, WI) was used to measure the level of hydrogen peroxide (H₂O₂), directly in cell culture according to the manufacturer's recommendations. Briefly, 80ul of cell suspension containing 5000 cells was seeded in a 96-well plate. After 24 hr pre-incubation at 37°C in 5% CO₂, the cells were incubated in room air (21% O₂, 5% CO₂) or hyperoxia (95% O₂, 5% CO₂). 20 µl of the H₂O₂ substrate solution was added to each well for the final 6 hr of treatment. 50 µl of ROS-Glo detection solution was then added and the plate was incubated for 20 minutes at room temperature and relative luminescence was recorded using a plate reader (M3 SpectraMax).

Tube Formation Assay

As a measure of angiogenesis *in vitro* we used the tube formation assay (ScienCell, Carlsbad, CA). Briefly, 150 µl of ECM Gel Solution was added to each well of 24-well plate and incubated 1 hour at 37°C to allow the ECM gel to solidify into a homogenous gel. 500 µl of HUVEC cells suspension at 0.5×10^5 cells/well were seeded into each well. The cells were incubated at 37°C in hyperoxia (95% O₂, 5% CO₂) for 18 hours. This duration of hyperoxia exposure to assess tube formation was used based on previous literature [11], in which the investigators used 20 hr of hyperoxia exposure. Moreover, we wanted to perform the functional assay before hyperoxia adversely affected cell viability. The development of tubular structure was analyzed in-situ with a phase-contrast microscope.

Real-time PCR

Total RNA was extracted from HUVECs and treated with DNase I (Invitrogen, Hercules, CA). cDNA was prepared by using iScript Advanced cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed using the ViiA7 real-time PCR detection system (Applied Biosystems, Foster City, CA) and SYBR Green (Bio-Rad, Hercules, CA). The thermal cycle condition used was as follows: 95 °C for 1 min, 40 cycles at 95 °C for 15 s, and 60 °C for 15 s. Primers used for quantitative PCR were as follows: VEGFR2 (forward): TGATCGGAAATGACACTGGA; (reverse): CACGACTCCATGTTGGTCAC; β-actin (forward): CATCGAGCACGGCATCGTCA; and

(reverse): TAGCACAGCCTGGATAGCAAC. Relative mRNA levels were calculated using the 2^{-CT} method. The DDCT method was used to calculate the fold change in mRNA expression: $DCt = Ct(\text{target gene}) - Ct(\text{reference gene})$, $DDCt = DCt(\text{treatment}) - DCt(\text{control})$, fold change = 2^{-DDCt}

Protein quantification

Total protein of HUVECs (baseline and after 48 h either in normoxia or after exposure to hyperoxia) was isolated using RIPA buffer containing protease inhibitors. The protein concentration was measured using the Bradford method. The protein concentration was determined using a standard curve built with different concentrations of BSA.

Western Immunoblotting

Total protein extract (20–30 μ g) was resolved by 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. The following primary antibodies were used: rabbit anti-VEGFR2 (1:1000, Cell Signaling, Beverly, MA), rabbit anti-IKK β (1:1000, Cell Signaling, Beverly, MA), rabbit anti-IKBA (1:1000, Cell Signaling, Beverly, MA), rabbit anti-p-IKBA (1:1000, Cell Signaling, Beverly, MA), and rabbit anti- β -actin (1:5000, Cell Signaling, Beverly, MA). ECL plus western blotting substrate was used for visualizing immunoreactive protein bands. The protein bands were normalized against β -actin (used as a loading control) on the same gel. Relative quantitation was performed using the Bio-Rad Image Analysis software with normalization against β -actin.

NF- κ B p65 ELISA Assay

NF- κ B p65 ELISA Assay (ENZO, Telluride, CO) was performed following the manufacturer instructions. Briefly, 50 μ l binding buffer was added to each well. 100 μ l of whole cell extract was added to each well and incubated for 1 hr at room temperature. After washing with 200 μ l wash buffer, 100 μ l NF- κ B p65 antibody (1:1000) was added to each well. The plate was incubated for 1 hr at room temperature. After washing again, 100 μ l of HRP-conjugated secondary antibody (1:10000) was added to each well. Following incubation and washing and addition of 100 μ l of substrate working solution to each well, the chemiluminescence was measured using a plate reader (M3 SpectraMax).

Statistical analysis

Data are displayed as mean $\pm$ SEM. Student's *t*-tests or two-way ANOVA following Bonferroni post-test was performed for statistical evaluation. In all analyses, significance level was set at $P < 0.05$.

Results

Sex-specific differences in cell viability, proliferation and oxidative stress in HUVECs exposed to hyperoxia

Male and female HUVECs were exposed to air or hyperoxia for up to 72 h, following which the cells were harvested to determine cell viability (Trypan blue assay) and cell proliferation

(CCK8 assay) and generation of H_2O_2 . Hyperoxia significantly decreased cell viability (Figure 1A) and proliferation (Figure 1B) in both male and female HUVECs compared to controls incubated in room air. Cell viability in male HUVECs decreased significantly compared to female HUVECs after 72 h of hyperoxia exposure. After 24 h and 48 h of hyperoxia treatment, both male and female HUVECs generated significantly more H_2O_2 than under the room air conditions (Figure 1C). The increase was greater in male HUVECs.

Effect of hyperoxia exposure on angiogenesis and differential modulation by sex

To measure differences in angiogenesis, we performed tube formation assay with male and female HUVECs under hyperoxic conditions. Upon exposure to hyperoxia, tube formation was severely impaired in male HUVECs. Tube length, total tubes/well, total branching points/well and total loops/well were significantly lower in male HUVECs compared to females (Figure 2). Representative phase contrast images are shown in Figure 2E and F.

Expression pattern of pro-angiogenic factor VEGFR2 in male and female HUVECs exposed to hyperoxia

At baseline, VEGFR2 mRNA levels were higher and protein levels were similar in male and female HUVECs. At 48h, in room air, male HUVECs had greater expression of VEGFR2 mRNA compared to female cells and the expression was increased in both male and female HUVECs from baseline. Hyperoxia exposure significantly decreased the expression in male HUVECs, but not in female HUVECs (Figure 3A). This decrease was also noted in VEGFR2 protein expression in male HUVECs but not in female cells. In room air (48h), there was increased expression of VEGFR2 protein in male HUVECs similar to the mRNA levels (Figure 3B).

Sex-specific modulation of the NF κ B pathway

Upon exposure to hyperoxia there was increased expression of IKK β in both male and female HUVECs, but it was greater in female HUVECs compared to males. IKK β expression was significantly higher in female HUVECs compared to males at 48h under hyperoxic conditions (Figure 4A). We also measured the expression of IK α and p-IK α expression in male and female HUVECs. IK α expression was similar in room air conditions and was increased at 48 hr under room air conditions in male HUVECs. The expression was greater in HUVECs exposed to hyperoxia compared to room air controls (Figure 4B). However, the expression of p-IK α was higher in female HUVECs at 48 h after exposure to hyperoxia compared to males. Also, upon exposure to hyperoxia, there was a significant increase from room air levels in female HUVECs (Figure 4C). We also measured the levels of active form of NF κ B p65 using ELISA. Under normal conditions, I κ B sequesters p65 or Rel A within the cytoplasm and this blocks NF κ B dependent transcription. Upon phosphorylation of I κ B, p65 is released and is free to associate with the p50 subunit. This leads to the translocation of the NF κ B complex into the nucleus and activation of NF κ B dependent genes. In our study, there was significant increase in the NF κ B pathway activation in female HUVECs at 48 h compared to male HUVECs (Figure 4D).

Discussion

In this study, we present the sex-specific differences in endothelial cell survival and function and the possible associated underlying mechanisms using male and female HUVECs in an *in vitro* model of oxygen toxicity.

Our laboratory has reported sex-specific differences in mortality and lung injury in an *in vivo* model for BPD. We exposed neonatal male and female mice (C57BL/6) to hyperoxia (95% FiO₂, PND 1-5). Alveolarization and pulmonary vascularization (vessel number and expression of markers of angiogenesis (PECAM1 and VEGFR2) was impaired to a greater extent in males. Macrophage and neutrophil infiltration was significantly increased in hyperoxia-exposed animals but was increased to a greater extent in males compared to females [9].

Other investigators have described sex-specific differences in endothelial cell properties and function. *Addis et al.* showed that cell proliferation, migration, nitric oxide synthase 3 expression were higher in female HUVECs even at baseline [6]. *Lorenz et al.* showed that female HUVECs have higher cell viability after serum starvation and an increased tube formation capacity compared to male cells [12]. In our study, we show improved cell viability and tube formation capacity in female HUVECs even after exposure to hyperoxia. Sexually dimorphic differences in prostacyclin and prostaglandin synthesis [13] and susceptibility to oxidative stress and apoptosis induced by specific autoantibodies [14] have also been reported. Dimorphic transcriptional differences were observed between male and female HUVECs in response to shear stress with greater expression of immune responsive genes in female cells [12]. Similar results were reported by Penaloza et al in fetal cells before exposure to fetal sex hormones [15]. The effect of male and female sex hormones on HUVECs has been extensively studied to elucidate mechanisms behind sex-specific morbidities in cardiovascular disease. For example, androgens have been shown to have a pro-inflammatory effect by amplifying the effect of TNF- α in both male and female HUVECs [16].

In this study, oxidative stress as measured by H₂O₂ production, was greater in male HUVECs compared to females. Increase in H₂O₂ in an *in vitro* model of pulmonary oxygen toxicity has been reported previously [10]. Better antioxidant defense mechanisms have been reported in both female human neonates and murine studies, compared to males [17,18], [19]. Another study reported that the enzymes involved in glutathione synthesis; glutathione peroxidase and regeneration; glutathione reductase were higher in females in venous blood samples [20]. Decreased oxidative stress could have contributed to the survival advantage in female HUVECs observed in our study.

Exposure to hyperoxic conditions postnatally leads to alveolar hypoplasia and abnormal growth of the lung vasculature[21]. In this study, we show that female HUVECs had better preserved angiogenic function after exposure to hyperoxia compared to males. Vascular endothelial growth factor (VEGF) plays a key role in lung development. It acts through two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/flk-1). In the developing lung, VEGF is expressed at the distal tips of the developing lung buds and its receptor;

VEGFR-2 is expressed on the endothelium developing around these developing airways [21]. Maniscalco *et al.* showed that, in neonatal rabbits exposed to 100% O₂ for 9 days, there was decreased VEGF mRNA expression and VEGF immunostaining in the lung [22]. A similar response was seen with VEGF receptors in newborn rats [23]. In our model, hyperoxia significantly decreased VEGFR2 expression at 48 h but the decrease was greater in male HUVECs. At 48 hours under room air, there was increase in VEGFR-2 expression over baseline (0 h) in both male and female HUVECs. This may be related to increased expression of VEGFR2 in cells maintained under optimal culture conditions. This pattern has been reported previously by other investigators [24,25].

The NF- κ B heterodimer; p50/p65(RELA) [p65 acts as a transactivator whereas p50 possesses DNA binding activity] is inhibited by I κ B proteins and sequestered in the cytoplasm. I κ B, thus acts as a key regulatory molecule and blocks NF κ B dependent transcription. Phosphorylation of I- κ B proteins by I Kappa Kinases (IKK), which include IKK α and IKK β , targets them for degradation. p65 is then released and free to associate with the p50 subunit; releasing the active NF- κ B to enter the nucleus and activate gene expression [26]. Sustained activation of NF- κ B has been shown to attenuate hyperoxia-induced mortality in adults and improve survival, preserve lung development and decrease hyperoxic lung injury in neonatal mice [27]. In human pulmonary epithelial cells, NF- κ B pathway activation decreased hyperoxia induced cell death [28]. NF- κ B was a direct regulator of VEGFR2 in the neonatal pulmonary vasculature and its inhibition decreased angiogenesis [29]. Pro-angiogenic properties of NF- κ B have been reported in other disease models [30] [31] [32]. We show in our study that the NF- κ B pathway activation is greater in female HUVECs compared to male with greater expression of IKK β , p-IK β α and greater level of p65 activation. This finding, accompanied with the greater decline in VEGFR2 expression in male HUVECs, may explain the better preservation of angiogenesis in female HUVECs. Similarly, we have previously reported that when neonatal mice were exposed to postnatal hyperoxia, female mice showed increased activation of the NF- κ B pathway *in vivo* in the lungs compared to males [9].

In conclusion, we show that sex differences are present in male and female HUVECs in their responses to hyperoxia *in vitro*. Since endothelial dysfunction has a major role in the pathogenesis of BPD, these differences could explain in part the mechanisms behind sex-specific differences in the incidence of this disease among preterm neonates and may aid in the development of individualized therapies to prevent/treat BPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Cellular sex effects viability and oxidative stress in HUVECs exposed to hyperoxia
2. Male HUVECs show greater impairment in angiogenesis compared to female cells
3. Sex-specific modulation of VEGFR2 and the NF-kappaB pathway was noted

Figure 1.

Effects of hyperoxia on cell viability, proliferation, and reactive oxygen species (H_2O_2) production in male and female HUVECs. Male and Female HUVECs exposed to room air (RA) (room air-5% CO_2) and 24, 48, or 72 h of hyperoxia (HO) (95% O_2 -5% CO_2) were subjected to trypan blue exclusion (A) or CCK8 assay (B) or ROS-GloTM luminescent H_2O_2 assay to measure the oxidative stress (C) as described in materials and methods. Values are means $\pm$ SEM. Significant differences between baseline and subsequent time-points within the same sex are indicated by ** $P < 0.01$ and *** $P < 0.001$. Significant differences between male and female HUVECs at each time-point is indicated by ††† $P < 0.001$ (male versus female at certain time point).

Figure 2.

Effect of cell sex on tube formation assay. Male and Female HUVECs exposed to 18 h of hyperoxia (95% O₂-5% CO₂) were subjected to tube formation assay as a measure of angiogenesis. Tube length (A), total tubes/well (B), total loops/well (C) and total branching points/well (D) were quantitated. Significant differences between male and female HUVECs are indicated by *** P<0.001. Representative phase contrast images are shown in Figures 3E (male) and 3F (female). Values are expressed as mean ± SEM. N=6 for each group.

Figure 3.

VEGFR2 mRNA and protein expression in male and female HUVECs. Male and Female HUVECs exposed to room air (RA) (room air-5% CO₂) and 48 h of hyperoxia (HO) (95% O₂-5% CO₂). VEGFR2 mRNA (A) and protein (B) were measured. Representative western blot images and densitometry analyses are shown. Values are expressed as mean $\pm$ SEM. N=6 for each group. Significant differences between the indicated groups are represented by * $P < 0.05$, *** $P < 0.001$.

Figure 4.

Sex-specific differences in modulation of NF κ B pathway. Male and Female HUVECs exposed to room air (RA) (room air-5% CO₂) or hyperoxia (HO) (95% O₂-5% CO₂ for 48h. Expression of IKK β (A), IKB α (B) and p-IKb α (C) were measured. Representative western blot images and densitometry analyses are shown. Levels of active form of NF κ B p65 were measured using ELISA (D) (0, 8, 24 and 48h). Values are expressed as mean $\pm$ SEM. N=6 for each group. Significant differences between the indicated groups are represented by * P < 0.05, ** P < 0.01. Significant difference between male and female HUVECs is represented by † P < 0.05 for the NF- κ B p65 ELISA study and by *** P < 0.001 for significant differences from baseline values.