Bioresearch Communications
Volume 02, issue 01, July 2016.

Journal Homepage: www.bioresearchcommunications.com

Bioresearch Communications

Original Article

Hypoxia modulates the cellular signaling in cultured ratlung micro vascular endothelial cells

Badri Giri, MD[1], Syeda H Afroze, PhD[2], Saiara Choudhury, BS[3], Raina Karanjeet, MD[4], Shekhar A. Ghamande, MD[1,3], David C Zawieja, PhD[2], M. Nasir Uddin, PhD, FAHA[*3,4,5,6]

Departments of Pulmonary and Critical Care[1], Medical Physiology[2], Internal Medicine[4], Obstetrics & Gynecology[5], and Pediatrics[6] [3]Texas A&M Health Science Center College of Medicine Baylor Scott & White Health and Texas A&M Health Science Center College of Medicine, Temple, Texas 76508

ABSTRACT: Acute and chronic oxidative stress is one of the causative factors for endothelial dysfunction; however, itsunderlying mechanism is still unknown. In this study, alterations in signaling proteins in hypoxia-induced rat lung microvascular endothelial cells (RLMEC)were identifiedin vitro. RLMEC were maintained in hypoxia (2% O_2) for 24 hours and 48 hours, as well as in hyperoxia (20% O_2) and normoxia (5% O_2). Some cells were incubated in normoxia following exposure to hypoxia. Western Blotwas performed to determine expression of the signaling proteins. The data were statisticallyanalyzed. Hypoxia induced the up-regulation of p38 MAPK, Cox-2, Bax/Bcl-2, AT_1, IL-6 and down-regulatedeNOS in RLMEC compared to normoxia.Additionally, RLMEC incubated at hypoxic condition for 48 hours caused significantly higher expressions of apoptotic and stress signaling proteins compared to 24hour incubation. Thesignaling was reversed in cells that were exposed to 24hourshypoxic condition followed by 24hours innormoxia; however, it was not reversed after a 48 hour hypoxia induction.Conclusion: Hypoxia induces up-regulation of stress and apoptotic signaling in cultured RLMEC that may indicate endothelial dysfunction. This in vitro system can be used as a tool to study hypoxia-induced endothelial dysfunction in diseased condition.

CITATION: Giri, B., Afroze, S. H., Choudhury, S., Karanjeet, R., Ghamande, S. A., Zawieja, D. C., Uddin, M. N. 2016. Hypoxia modulates the cellular signaling in cultured ratlung micro vascular endothelial cells. *Biores Comm*. 2(2), 230-237.

KEYWORDS: Endothelial Cells; Hypoxia; Stress signaling; Apoptosis;Inflammation

CORRESPONDENCE: M. Nasir Uddin, E-mail: Mohammad.uddin1@BSWHealth.org

Significance of the paper: Pulmonary endothelial dysfunction is a common occurrence in many pulmonary diseases; however, itsunderlying mechanism is still unknown. This in vitro study is intended to understand the molecular and cellular signaling in hopxia-induced cultured endothelial cells. The outcome of the study would be used as a tool to study hypoxia-induced endothelial dysfunction in diseased condition.

Introduction:

Pulmonary endothelial dysfunction is a common occurrence in many pulmonary diseases[1-4]. The normal function of pulmonary endothelium includes maintenance of vascular tone, homeostasis, leukocyte trafficking, transduction of luminal signals to abluminal vascular tissues, production of growth factors and cell signals with autocrine and paracrine effects and barrier function[5]. The normal endothelium is a "quiescent" cell line. Once it is "activated" by any condition, these cells release diverse growth factors and vasoactive mediators, which regulate the physical and biochemical properties of the pulmonary vessels and affect vascular contractility and cell growth. In healthy individuals, a balance between these mediators is thought to mediate the low basal pulmonary vascular tone, homeostasis, and vascular injury repair and growth[6]. Pulmonary endothelium varies from systemic endothelium in ultrastructure and function, and also differs in various vessel types in the pulmonary vasculature [7].

Hypoxia, or low oxygen tension,has been shown to alter endothelial cell physiology *in vivo* and *in vitro* in a number of ways. It can transcriptionally modulate gene expression of vasoactive mediators, promote angiogenesis and inhibit expression of vasodilators[8]Akhmeneeva found that hypoxia causes transcriptional induction of genes that encode vasoconstrictors and smooth muscle mitogens, such asendothelin-1and thrombospondin-1 and it also causes the reciprocal transcriptional inhibition of endothelial nitric oxide synthase (eNOS)[9].Additionally, Graven et al demonstrated that, when cultured endothelial cells are exposed to hypoxic conditions, they up-regulate the expression of a set of stress proteins called hypoxia-associated proteins (HAPs)[10]. Furthermore, Urban et al demonstrated that expressions of other HAPs by endothelial cells are directly related to the ability of endothelial cells to adapt to hypoxia[11], thus, leading to endothelial dysfunction.It has been shown that pulmonary endothelial dysfunction is associated with various pulmonary pathologies such as, adult respiratory distress syndrome (ARDS), pulmonary arterial hypertension (PAH), obstructive sleep apnea (OSA), chronic obstructive pulmonary disorder (COPD), Ventilator associated lung injury (VALI) and asthma[1-5,12]. Previous studies have identified many proteins that have an altered expression in hypoxic conditions. However, the pathophysiology of endothelial dysfunction in these diseases is not well known.

For exampleincreased levels of IL-6, decreased levels of angiotensin II Type 1 receptor (AT_1) and eNOS have been reported in endothelia dysfunction inpatients with OSA [1].Studies have demonstrated modulation of expression of eNOS in COPD patients and increased levels of IL-6 have been demonstrated in patients with VALI and asthma, as well [4,12]. While these studies identify biomarkers that span a variety of respiratory disorders, all of their pathologies induce conditions of chronic intermittenthypoxia and oxidative stress in the cells which have been related topulmonary endothelial dysfunction[13]. We believe that, in addition to the proteins that have been identified to have an altered expression, there are other proteins that undergo change in expression in pulmonary endothelial cells in hypoxia that could lead to endothelial dysfunction. Hypoxia has been shown to cause an increase in stress signaling proteins, apoptotic proteins and proinflammatory cytokines that can lead to aberrant inflammation. For example, Usatyuk et al have shown that reactive oxygen species(ROS) induced p38 mitogen activated kinase (MAPK) activationincreases endothelial permeability and causes endothelial barrier dysfunction in lung microvascular endothelial cells in ARDS[14].Additionally, IL-1 and IL-6 have been shown to mediate endothelial dysfunction in ARDS and VALI, and PAH and OSA, respectively[12,15,16].

Thus, our study observes the expression of proteins we suspect would be affected during hypoxia and can be used as biomarkers for pulmonary endothelial dysfunction. The expression of proteins that have been analyzed areBcl-2-associated X protein (Bax), Bcl-2 protein, p38 mitogen-activated protein kinase (p38 MAPK), cyclooxygenase-2 (Cox-2), angiotensin II type 1 receptor(AT_1),eNOS and IL-6 in rat lung micro vascular endothelial cells (RLMEC)*in vitro* in response to being exposed to hypoxic conditions.

Materials and Methods:

Cell Culture

Rat lung microvascular endothelial cell (RLMEC) utilized in this studywas cultured on fibronectin-coated dishes in complete MCDB-3 media,and supplemented with 10% fetal bovine serum (FBS)and endothelial cell growth medium-2 (EGM-2) SingleQuot Kit Supplement & Growth Factors (Lonza, Switzerland), which contains 2% FBS, VEGF (vascular endothelial growth factor), hFDF-B, R3-IGF-1, ascorbic acid, heparin, GA-1000 (Gentamicin and Amphotericin-B), hydrocortisone, hEGF and No BBE (Bovine Brain Extract). Cells were incubated at 37°C, 5% CO2 and 99% humidity (Fischer, Isotemp CO2 Incubator).

Hypoxia Exposure:

To vary the amount of oxygen the cells were exposed to, cells were grown separately in normal conditions (5% O_2) for 48 hours; hyperoxic conditions (20% O_2) for 48 hours and hypoxic conditions (2% O_2) for 24 hours and 48 hours, respectively. Some cells were incubated in normal condition for 24 hours and 48 hours and then exposed to hypoxia for 24hours and 48hours, respectively. Varying oxygen levels were controlled by changing O_2 levels in the incubator.

Western Blot Analysis for p38 MAPK, Bax, Bcl-2 protein, Cox-2, eNOS and AT_1:

After hypoxic exposure,the cell lysates were collected by a cell lysis buffer (Cell Signaling Technology) containing 0.1M Tris at pH 7.4, 50M NaCl, 0.5M EDTAat pH 8, Igepal, water and protease inhibitor cocktail (PIC). Protein concentrations were determined by using a BCA Protein Assay Kit(Pierce, Rockford, IL). An equal amount of protein was separated from each

sample using NuPage Novex 4-12% Bis-Tris gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were afterwards blocked in 5% milk probed with anti-p38 MAPK, Bax, Bcl-2, eNOS, AT_1, Caspase-9 or Cox-2 antibodies in separate experiments, respectively.After incubation with their corresponding secondary antibodies, proteins were visualized with a chemiluminescence detection system (Pierce). The intensity of the bands was determined using ImageQuant LAS 4000 (GE Healthcare, Life Sciences). The expressionsof p38 MAPK, Bax, Bcl-2, Cox-2, eNOS and AT_1 were quantified by densitometry analysis using Image J software(National Institutes of Health, Bethesda, MD) where the target protein was normalized to a structural protein (β-actin) to control between groups. This ensured correction for the amount of total protein on the membrane. Then the phospho-p38 was normalized to total p38. IL-6 was separately measured in the cell culture media by a commercially available ELISA assay kit.

Statistical Method

Data is expressed as mean ± SE (standard error). For statistical analyses, unpaired Duncan's post hoc test was applied; $p < 0.05$ was considered as statistically significa

Results

Hypoxia induced the up-regulation of stress signaling, p38 MAPK. In the Western Blot assay, cells grown in hypoxic conditions were observed to have a 1.5 fold increased expression of p38 MAPK compared to cells in normoxic and hyperoxic conditions (Fig 1A, B).

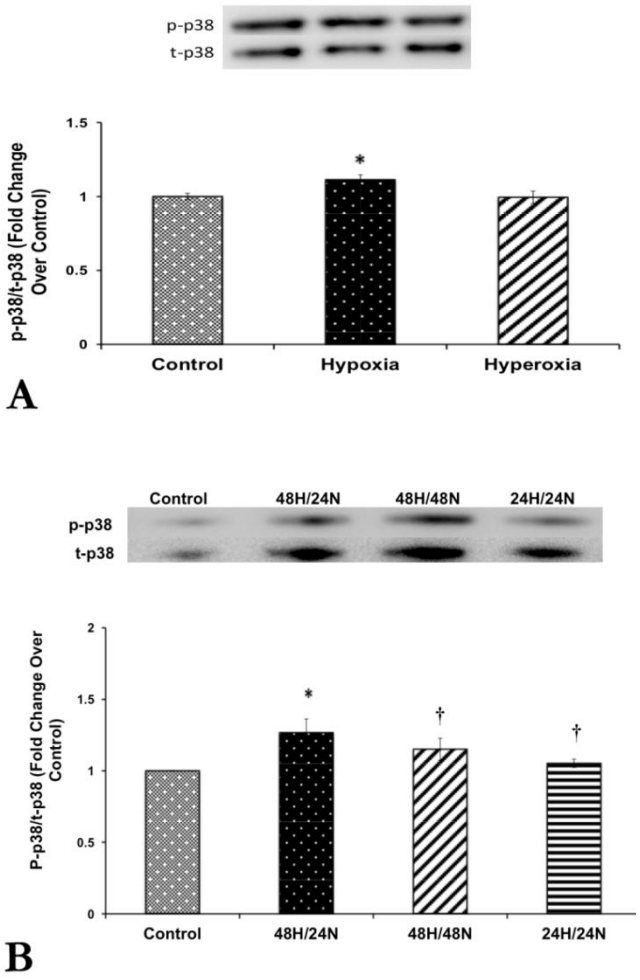


Fig 1 A & B. Hypoxia induced the up-regulation ($p<0.05$) of stress signaling (p38 MAPK (1.5 fold change).

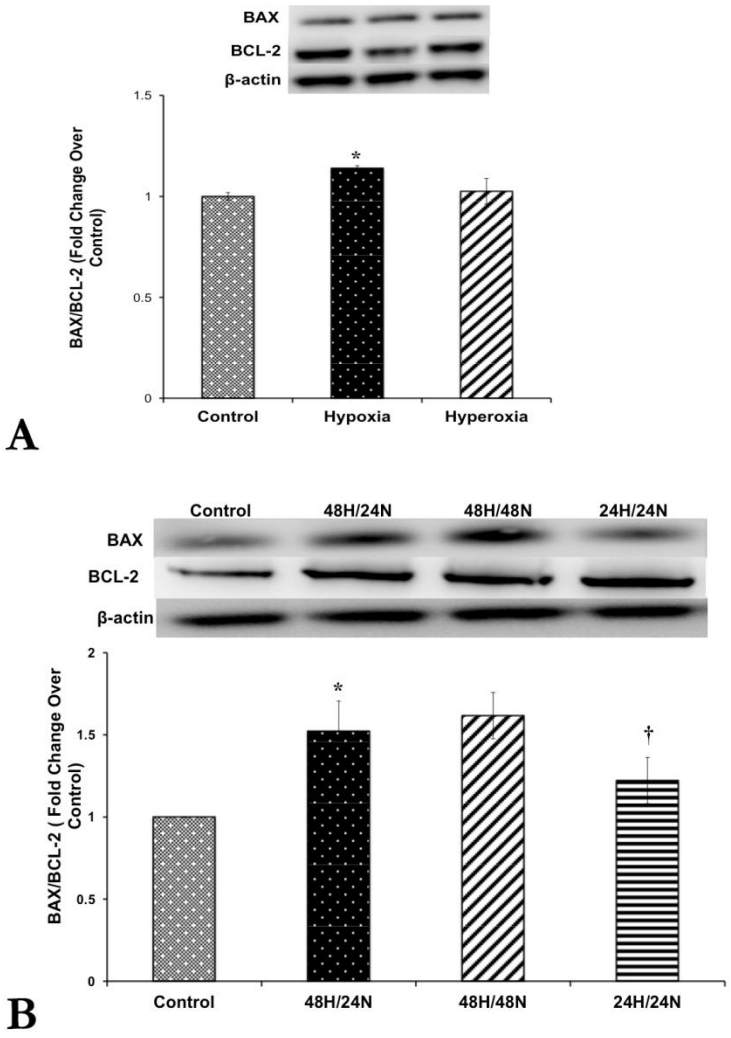


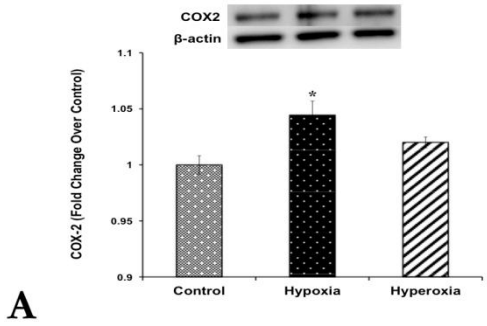
Fig 2 A & B. Hypoxia up-regulated pro-apoptotic signaling BAX/BCL-2 (1.4 fold change) and effect was reversible for the 24 hour period hypoxia.

Hypoxia up-regulated pro- apoptotic signaling

Compared to normoxic conditions, cells in hypoxic conditions had a 1.4 fold increased expression of Bax/Bcl-2. This effect was reversible for the 24 hour period hypoxia (Fig 2 A, B)

Hypoxia induced the up-regulation ($p<0.05$) of stress signaling Cox-2

Compared to normoxic conditions, cells in hypoxic conditions had a 1.4 fold increased expression of Cox-2 (Fig 3 A, B).



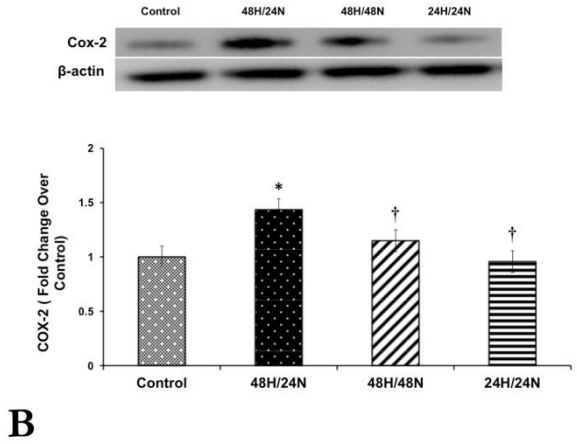


Fig 3 A & B. Hypoxia induced the up-regulation ($p<0.05$) of stress signaling Cox-2, (1.4 fold change).

Hypoxia induced the down-regulation ($p<0.05$) of eNOS expression

Compared to normoxic conditions, cells in hypoxic conditions had a decrease in eNOS expression (Fig 4). However, eNOS was found to be reversible to normal by 48 hour exposure to normoxic conditions after 48 hoursin hypoxic condition.

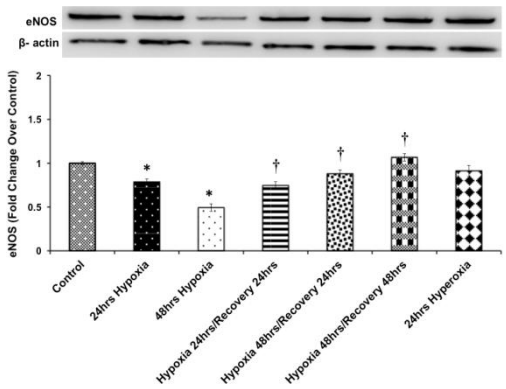
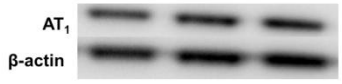


Fig 4: The expression of eNOS ($p<0.05$) was down-regulated in hypxic condition. The 48 hour hypoxia induced higher expression of apoptotic and stress signaling proteins compared to 24 hour. The cells that were exposed to normoxia for 24hours following the 24hour hypoxic condition showed a reversal of the signaling. However, those were not reversed by 24hour or 48hour exposure to normoxia after 48 hour hypoxia. In contrast, eNOS was found to be reversible to normal by 48 hour exposure to normoxia after 48 hour hypoxic condition.

Hypoxia induced the up-regulation ($p<0.05$) of AT_1

Compared to cells in normoxic conditions, cells in hypoxic conditions had a 1.3 fold increased expression of AT_1 (Fig 5).



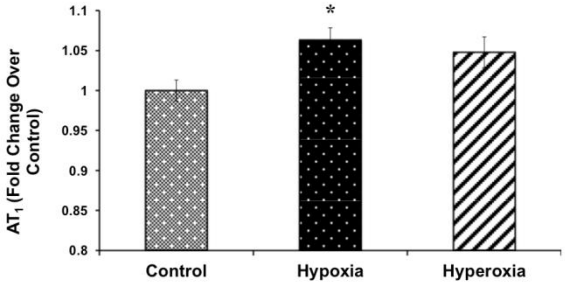


Fig 5. The vasoconstrictor receptors AT_1 were upregulated (1.3 fold change) in RLMEC compared to normoxia.

Hypoxia induced the up-regulation ($p<0.05$) of IL-6 secretion

In both 24hour and 48hour hypoxia exposure,

IL-6 secretion by RLMEC was up-regulated(Fig 6). Both the cells that were exposed to normoxia for 24hoursfollowed by 24 hours in hypoxia and cells that were exposed to normoxia for 48hours following 48hour hypoxic condition showed an upregulation during ormoxia and downregulation during hypoxiaof IL-6 secretion by RLMEC.

Additionally, hypoxic conditions for 48hours induced a higher expression of apoptotic and stress signaling proteins compared to that for 24hours. The cells that were exposed to normoxia for 24hours following 24hour hypoxic condition showed a reversal of the apoptotic signaling. However, cells that were initially exposed to 48 hour hypoxia period followed by 24hours or 48hours exposure to normoxiadid not show a reversal in their cellular signaling.

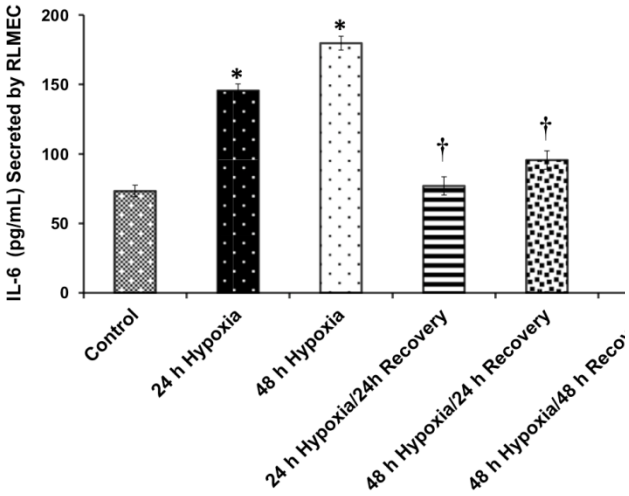


Fig 6.IL-6 secretion by RLMEC was upregulated by both 24 and 48 hour hypoxia exposure. The cells that were exposed to normoxia for 24 and 48 hour following the 24 and 48 hour hypoxic condition respectively, showed a reversal of the IL-6 secretion by RL.

Discussion:

Since pulmonary endothelial cells have a critical role in gas exchange, it is vital to continue investigation into the pathophysiology of endothelial dysfunction and the specific role that signaling proteins have in the process. Our results show that hypoxic conditions cause a significant change in the expression of many signaling proteins that can contribute to endothelial dysfunction.An increase in the expression of Bax/Bcl-2 in hypoxia indicates that apoptotic signaling may play an important role in endothelial dysfunction. This is consistent with a study mentioned previously[17] and shows that not only do increases in apoptotic proteins increase in OSA, but other hypoxia inducing conditions as well.Similarly, up-regulation of p38 MAPK and Cox-2 indicate that stress signaling also plays a part in pulmonary endothelial dysfunction. Moreover, our results show a down-regulation in eNOS expression. This is consistent with previous studies that have shown that pulmonary vascular responses to hypoxia in mice with targeted disruption of the eNOS gene ($eNOS^{-/-}$). Marked PH was found in $eNOS^{-/-}$ mice that grew in mild hypoxia when compared with the controls or $eNOS^{-/-}$ mice that grew in conditions simulating sea level[18]. Additionally, Ozaki *et al* demonstrated in their study that overproduction of eNOS in transgenic mice prevents hypoxia-induced PAH[19]. eNOS catalyzes the conversion of L-arginine to citrulline[20]. This produces nitric oxide (NO) that can act as a vasodilator on smooth muscle cells. According to Faller, NO can affect signals induced by hypoxia and vice versa[8]. This means that in hypoxic conditions, eNOS and hence, NO synthesis should decrease resulting in inhibition of vasodilation, thus causing vasoconstriction[8]. Furthermore, our results also show increased expression of AT_1. This is also consistent because AT_1 is a vasoconstrictor and its level increased in cases of COPD[21] and PAH[22]. These indicate that there might be a decrease in vasodilation and an increase in vasoconstriction involved in the physiology of endothelial dysfunction in hypoxic stress. Finally, IL-6 up-regulation is consistent with previous studies of known intermittent hypoxiainducible conditions[1,4,5,9,12,16] and indicates that aninflammatory process is involved in the pathogenesis of endothelial dysfunction,as well.

Furthermore, our experimental results show that reversal of signaling protein expression is possible in some cases, depending on time of exposure of cells to hypoxic and then normoxic conditions. This is very important since a number of pathologies, including, but not limited to, OSA, PAH, VALI, COPD, asthma and ARDS can cause intermittent hypoxic conditions that can be reverted back to normoxic conditions using an oxygen supplementation or other measures like treatment of OSA with positive airway pressure. Previously, Urban et al had shown endothelial dysfunction by the measurement of flow mediation dilation technique (FMD) during acute exacerbation of COPD. They studied 29 patients with moderate to severe COPD and found

mean FMD 6.7+/-3.6% at the time of exacerbations, which later improved to 10+/-3.4%, p<0.001 after clinical recovery [11]. Thus, our results indicate that depending on the length of exposure to hypoxia, therapeutic intervention may be implemented at a particular time point that can ensure endothelial cell recovery.

While our study identifies a few general cell signaling proteins that can be used to identify pulmonary endothelial dysfunction in hypoxic conditions, it is important to continue investigation to learn how these markers alter cell signaling that contribute to endothelial dysfunction and cause specific disease pathologies.This will help to identify specific points of therapeutic intervention for a particular respiratory disease that can be implemented on patients suffering from that specific disease.

Funding: Funding for this work was provided by Scott, Sherwood and Brindley Foundation, Scott & White Healthcare and from the Baylor Scott & White Research & Education Division (MNU), Temple, Texas.

Acknowledgements: The authors thank Glen Cryer and Chandler W Carroll for assistance with manuscript preparation.

Conflict of interest: The authors have read the journal's policy on conflicts of interest and the authors have no conflicts of interest to declare. All authors have read the journal's authorship agreement.

Ethical approval: This article does not contain any studies with animal and human participants performed by any of the authors.

References:

1. Budhiraja, R., Parthasarathy, S., and Quan S.F. 2007. Endothelial dysfunction in obstructive sleep apnea. *J. Clin. Sleep. Med.***3**:409-415.
2. Haslip, M., Dostanic, I., Huang, Y., Zhang, Y., Russell, K.S., Jurczak, M.J., Mannam, P., Giordano, F., Erzurum, S.C., and Lee, P.J. 2015.Endothelial uncoupling protein 2 regulates mitophagy and pulmonary hypertension during intermittent hypoxia. *Arterioscler. Thromb. Vasc. Biol.***35**:1166-1178.
3. Moussa, M.D., Santonocito, C., Fagnoul, D., Donadello, K., Pradier, O., Gaussem, P., De backer, D., and Vincent, J.L. 2015.Evaluation of endothelial damage in sepsis-related ARDS using circulating endothelial cell. *Intensive. Care. Med.***41**:231-238.
4. Wanner, A., and Mendes, E.S. 2015. Airway endothelial dysfunction in asthma and chronic obstructive pulmonary disease: a challenge for future research. *Am. J. Respir. Crit. Care. Med.***182**:1344-1351.
5. Budhiraja, R., Tuder, R.M., and Hassoun, P.M. 2004.Endothelial dysfunction in pulmonary hypertension. *Circulation*.**109**:159-165.
6. Chichger, H., Braza, J., Duong, H., and Harrington, E.O. 2015. SH2 domain-containing protein tyrosine phosphatase 2 and focal adhesion kinase protein interactions regulate pulmonary endothelium barrier function. *Am. J. Respir. Cell. Mol. Biol.***52**:695-707.
7. Aird, W.C. 2015.Endothelial cell heterogeneity. *Crit. Care. Med.***31**:S221-230.
8. Faller, D.V.1999. Endothelial cell responses to hypoxic stress. *Clin. Exp. Pharmacol. Physiol.***26**:74-84.
9. Akhimineeva, A.Kh. 2014. [Biochemical markers of endothelial dysfunction in chronic obstructive pulmonary disease concurrent with hypertensive disease or coronary heart disease]. *Ter. Arkh.***86**: 20-23.
10. Graven, K.K. and Farber, H.W. 1998. Endothelial cell hypoxic stress proteins. *J. Lab. Clin. Med.***132**:456-463.
11. Urban, M.H., Valipour, A., Kiss, D., Eickhoff, P., Funk, G.C., and Burghuber, O.C. 2014. Soluble receptor of advanced glycation end-products and endothelial dysfunction in COPD. *Respir. Med.***108**:891-897.

12. Gurkan, O.U., He, C., Zielinski, R., Rabb, H., King, L.S., Dodd-o, J.M., D'Alessio, F.R., Aggarwal, N., Pearse, D., and Becker, P.M. 2011. Interleukin-6 mediates pulmonary vascular permeability in a two-hit model of ventilator-associated lung injury. *Exp. Lung. Res.***37**:575-584.
13. Marcus, N.J., Philippi, N.R., Bird, C.E., Li, Y.L., Schultz, H.D., and Morgan, B.J. 2012. Effect of AT1 receptor blockade on intermittent hypoxia-induced endothelial dysfunction. *Respir. Physiol. Neurobiol.***183**:67-74.
14. Usatyuk, P.V., Vepa, S., Watkins, T., He, D., Parinadi, N.L., and Natarajan, V. 2003. Redox regulation of reactive oxygen species-induced p38 Map kinase activation and barrier dysfunction in lung microvascular endothelial cells. *Antioxid. Redox. Signal.***5**: 723-730.
15. Good, R.B., Gilbane, A.J., Trinder, S.L., Denton, C.P., Coghlan, G., Abraham, D.J., and Holmes, A.M. 2015. Endothelial to mesenchymal transition contributes to endothelial dysfunction in pulmonary hypertension. *Am. J. Pathol.***185**: 1850-1858.
16. Mokra, D., and Kosutova, P. 2015. Biomarkers in acute lung injury. *Respir. Physio. Neurobiol.* **209**: 52-58.
17. El Solh, A.A., Akinnusi, M.E., Berim, I.G., Peter, A.M., Paasch, L.L., and Szarpa, K.R. 2008. Hemostatic implications of endothelial cell apoptosis in obstructive sleep apnea. *Sleep. Breath.***12**:331-337.
18. Fagan, K.A., Fouty, B.W., Tyler, R.C., Morris, K.G., Jr., Hepler, L.K., LeCras, T.D., Abman, S.H., Weinberger, H.D., Huang, P.L., McMurtry, I.F., et al. 1999. The pulmonary circulation of homozygous or heterozygous eNOS-null mice is hyperresponsive to mild hypoxia. *J. Clin. Invest.***103**:291-299.
19. Ozaki, M., Kawashima, S., Yamashita, T., Ohashi, Y., Rikitake, Y., Inoue, N., Hirata, K.I., Hayashi, Y., Itoh, H., and Yokoyama, M. 2001. Reduced hypoxic pulmonary vascular remodeling by nitric oxide from the endothelium. *Hypertension.***37**:322-327.
20. Mehta, S., Steward, D.J., Langleben, D., and Levy, R.D. 1995. Short-term pulmonary vasodilation with L-arginine in pulmonary hypertension. *Circulation*.**92**:1539-1545.
21. Bullock, G.R., Steyaert, I., Bibe, G., Carey, R.M., Kips, J., De Paepe, B., Pauwels, R., Praet, M., Siragy, H.M., and de Gasparo, M. 2001. Distribution of type-1 and type-2 angiotensin receptors in the normal human lung and in lungs from patients with chronic obstructive pulmonary disease. *Histochem. Cell. Bio.***115**:117-124.
22. Giaid, A., and Saleh, D. 1995. Reduced expression of endothelial nitric oxide synthases in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.***333**:214-221.