Disappearance of Hypoxic Pulmonary Vasoconstriction and O2-Sensitive Nonselective Cationic Current in Arterial Myocytes of Rats Under Ambient Hypoxia

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Acute hypoxia induces contraction of pulmonary artery (PA) to protect ventilation/perfusion mismatch in lungs. As for the cellular mechanism of hypoxic pulmonary vasoconstriction (HPV), hypoxic inhibition of voltage-gated K⁺ channel (Kᵥ) in PA smooth muscle cell (PASMC) has been suggested. In addition, our recent study showed that thromboxane A₂ (TXA₂) and hypoxia-activated nonselective cation channel (Ιₛₘₖ) is also essential for HPV. However, it is not well understood whether HPV is maintained in the animals exposed to ambient hypoxia for two days (2d-H). Specifically, the associated electrophysiological changes in PASMCs have not been studied. Here we investigate the effects of 2d-H on HPV in isolated ventilated/perfused lungs (V/P lungs) from rats. HPV was almost abolished without structural remodeling of PA in 2d-H rats, and the lost HPV was not recovered by Kᵥ inhibitor, 4-aminopyridine. Patch clamp study showed that the hypoxic inhibition of Kᵥ current in PASMC was similar between 2d-H and control. In contrast, hypoxia and TXA₂-activated Ιₘₖ was not observed in PASMCs of 2d-H. From above results, it is suggested that the decreased Ιₘₖ might be the primary functional cause of HPV disappearance in the relatively early period (2 d) of hypoxia.

Key Words: Chronic hypoxia, Hypoxic pulmonary vasoconstriction, Nonselective cation channel, O₂-sensitive ion channel, Pulmonary artery

INTRODUCTION

Hypoxic pulmonary vasoconstriction (HPV) is an acute physiological response of pulmonary arteries (PAs) to poorly ventilated alveolar regions, thereby prevents arterial hypoxemia by optimizing ventilation-perfusion ratio in lungs. As for the underlying cellular mechanisms for HPV, numerous evidences support that O₂-sensitive ion channels play critical roles. For example, hypoxic inhibition of voltage-gated K⁺ channels (Kᵥ) in pulmonary arterial smooth muscle cells (PASMCs) has been regarded as a main mechanism by inducing membrane depolarization that subsequently activates L-type voltage-operated Ca²⁺ channels (VOCCL) [1-4]. In addition, our recent study in rat PA showed that a partial stimulation of thromboxane A₂ (TXA₂) receptor is required to effectively induce HPV [5]. U46619, a stable analogue of TXA₂, is commonly used as a vasoactive agent for studying HPV in isolated PAs. Electrophysiological investigation of rat PASMCs using whole-cell patch clamp technique demonstrated not only the hypoxic inhibition of Kᵥ current (Ιₖᵥ) but also the activation of nonselective cation channel (Ιₘₖ) by combined application of U46619 and hypoxia [5]. According to our previous report, both Ιₖᵥ inhibition and Ιₘₖ activation are required to induce sufficient depolarization of PASMCs, activating VOCCL and Ca²⁺ influx for contraction.

When animals are exposed to chronic hypoxia (CH), PAs undergo structural changes such as thickening in the medium. It has been reported that CH induces abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs), which is the cause of PA remodeling [6]. In addition to the structural changes, it has been reported that CH (for several weeks) markedly attenuates HPV in mammals including humans [7-9]. Interestingly, the functional changes, i.e. loss of HPV, seemed to occur at relatively early period of CH; two reports described that HPV was also weakened in rats those underwent 3 days of hypoxia [8,10]. These results suggest that the weakened oxygen sensitivity of PASMC might occur prior to PA remodeling.

According to previous studies of PASMCs from the long
term hypoxia animals (CH: 3 weeks, PO2 10%), the hypoxic inhibition of IKr was largely abolished, and the dysfunctional O2-sensitivity of Kv was suggested to underlie the attenuated HPV responses [9,11]. However, no previous study has investigated the electrophysiological changes of PASMCs from the animals exposed to the relatively short period of hypoxia (<3 days). Based on these backgrounds, here we investigate the changes of HPV and of the ion channel currents (IKr and INSC) in PASMCs from the rats exposed to two days of hypoxia (2d-H). Our present study demonstrates, for the first time, that the INSC induced by U46619 and hypoxia disappear at 2d-H while the hypoxic inhibition of IKr is unchanged.

**METHODS**

**Animal model for 2d-H**

All experimental procedures were performed with approval of the Institutional Animal Care and Use Committee (IACUC) in Seoul National University (IACUC approval no.: SNU 111129-1). Sprague-Dawley rats (230~260 g, male) were exposed to normobaric hypoxia chamber equipped with O2 sensor and automatic gas controller (ProOx Model 110, Biopsherix, USA). The hypoxia chamber could contain two regular rat cages. PO2 was initially set to 12% during the first digestion medium (Ca2+-free normal Tyrode’s (NT) solution including collagenase 2.5 mg/ml, bovine serum albumin (BSA) 1.5 mg/ml and dithiothreitol (DTT) 1.5 mg/ml) for 20 minutes, then moved to the second digestion medium (Ca2+-free NT solution including collagenase 2.5 mg/ml, BSA 1.5 mg/ml and DTT 1.5 mg/ml) and further incubated for 20 minutes at 37°C. The digested PASMCs were rinsed with a Ca2+-free NT solution and gently agitated using a Pasteur glass pipette. Cells were stored in Kraft-Brühe (KB) storage solution at 4°C until they are used for experiments in the same day.

**Measurement of membrane currents in Patch-clamp experiments**

Isolated cells were transferred to a bath chamber (approximately 0.2 ml) on the stage of an inverted microscope (IMT-2, Olympus, Osaka, Japan). In continuous perfusion with NT solutions (10 ml/min), whole cell and nystatin perforated patch clamp experiments were performed with patch-clamp amplifier (Axopatch-1D, Axon Instruments, Forster City, CA, USA) located in the perfused bath. 

**Histology**

Lungs were perfused with phosphate salt solution (PBS) and fixed by 4% paraformaldehyde for 24 hours, and then embedded in paraffin wax. For histological analysis, lung sections (5 μm) were cut and stained with hematoxylin and eosin (H&E). Stained lung tissues were analyzed by computerized image analysis system (Olympus BX51, Adobe Photoshop 7.0 software) at ×200 magnification.

**Preparations of isolated PASMCs**

The second and third branches of PASMCs (inner diameter 200~300 μm) were dissected under a binocular microscope. The dissected vessels were initially incubated in the first digestion medium [Ca2+-free normal Tyrode’s (NT) solution including papain 1 mg/ml, bovine serum albumin (BSA) 1.5 mg/ml and dithiothreitol (DTT) 1.5 mg/ml] for 20 minutes, then moved to the second digestion medium (Ca2+-free NT solution including collagenase 2.5 mg/ml, BSA 1.5 mg/ml and DTT 1.5 mg/ml) for 20 minutes at 37°C. The digested PASMCs were rinsed with a Ca2+-free NT solution and gently agitated using a Pasteur glass pipette. Cells were stored in Kraft-Brühe (KB) storage solution at 4°C until they are used for experiments in the same day.

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**Measurement of pulmonary arterial pressure (PAP) and HPV**

PAP and its increase in response to acute hypoxia (Δ PAPhypox) were measured in artificially ventilated and perfused lungs (V/P lungs). Rats were anesthetized with pentobarbital sodium (70 mg/kg, i.p. injection). To confirm appropriate anesthesia, reflexive eye blinking and loss of toe pinch were checked before experiment. After tracheostomy, rats were ventilated with a normoxic gas mixture (21% O2, 5% CO2 and 74% N2) via a rodent ventilator (respirator 645, Harvard Apparatus, USA). Ti dal volume (10 ml/kg) and ventilation (85 breaths/min) were consistently maintained. A median sternotomy was performed, and heparin (200 U/kg) was injected to protect blood coagulation. Then, a cannula was inserted into main pulmonary artery and the suture was tightened. To allow constant blood flow perfusion, another cannula was inserted into left atrium via the left ventriculotomy. The perfusate mixed with blood (20 ml of whole blood and 30 ml physiological salt solution, PSS) was recirculated (15 ml/min) via a peristaltic pump (Servo clamp experiments for recording NSC current, bath solutions and drugs

Physiological salt solution (PSS) for VP lungs contained the following (in mM): NaCl 131, KCl 4.7, MgSO4 1.17, NaHCO3 22.61, KH2PO4 1.18, Glucose 10, CaCl2 3.2, sodium mofenoanate 0.0050 g/l, bovine serum albumin (BSA) 30.0 g/l. The NT solution contained the following (in mM): NaNCl 140, KCl 5.4, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, NaH2PO4 0.33, glucose 10, CaCl2 1.8 and MgCl2 1 (adjusted with NaOH to pH 7.4). In Patch clamp experiments for recording NSC current, bath solution consisted of following compositions (in mM): NaCl 140, CaCl2 4, HEPES 10, Glucose 10, Sucrose 10, CaCl2 1.8 and MgCl2 0.5 (adjusted with NaOH to pH 7.4). The pipette solution for recording NSC current consisted of (in mM):
Fig. 1. Comparison of HPV, basal PAP and Ang II-induced \( \Delta \)PAP between control and 2d-H rats in V/P lungs. (A, B) Representative traces of PAP recording in control (A) and 2d-H rats (B). Hypoxic ventilation (3% PO\(_2\), 5 min) consistently increased PAP (\( \Delta \)PAP\(_{hypox}\)) implying HPV response (Fig. 1A, C, n=10). Interestingly, the HPV response was abolished in 2d-H rats, and did not recover by repeated application of hypoxia (Fig. 1B, C, n=10).

In addition, the initial transient increase of PAP in response to Ang II application was decreased by 55% in 2d-H rats (Fig. 1E). It has to be noted that the basal PAP of 2d-H rat was increased slightly but significantly (Fig. 1D).

Although not shown here, we also confirmed that HPV was completely abolished in the rats those have been exposed to hypoxia for 2 weeks and for 3 weeks (n=2; 2 weeks, n=4; 3 weeks). However, rats exposed to one day of hypoxia (12% PO\(_2\)) for 1 day still showed HPV response (n=3, data not shown).

Although the basal PAP was increased in 2d-H rats, right ventricular hypertrophy did not occur. Relative weights of right ventricle in comparison with left ventricle and septum (RV/(LV+S)) were analyzed, showing no difference between control and 2d-H rats (data not shown, n=10; control, n=5; 2d-H).

In the histological analysis of 2d-H and control lungs, there was no noticeable medial thickening of PA (Fig. 2A, B). In contrast, typical hypertrophic change of PA (medial thickening) was generally observed in rats those have been exposed to hypoxia for 2 and 3 weeks (Fig. 2C, D).

Previous studies have suggested that the inhibition of Kv channels by acute hypoxia (O\(_2\)-sensitivity of Kv) is critical to generate HPV, and the hypoxic inhibition of Kv was not observed in the 2- or 3-week CH models [4,7,8]. If the loss of O\(_2\)-sensitivity of Kv is also critical for the attenuation of HPV in our present study, we would expect that a pharmacological inhibition of Kv recovers HPV in 2d-H rats.

Application of 4-aminopyridine (4-AP, 5 mM) increased basal PAP and augmented HPV response in control rats (Fig. 3A). Similarly, basal PAP was also increased by 4-AP in 2d-H rats. However, 4-AP did not restore the attenuated HPV (Fig. 3B). Summarized data are shown in Fig. 3C.
Fig. 3. Effect of 4-AP, a Kv inhibitor, on PAP and HPV ($\Delta$PAP$_{\text{hypoxia}}$) in control and 2d-H rats. (A, B) Representative traces of PAP recording in control and 2d-H rats. Both basal PAP and $\Delta$PAP$_{\text{hypoxia}}$ were increased by 5 mM 4-AP in control (A). In 2d-H, however, basal PAP was slightly increased by 4-AP, and $\Delta$PAP$_{\text{hypoxia}}$ was not recovered by the 4-AP pretreatment (B). (C) Summaries of the results shown as bar graphs (n=5; control, n=4; 2d-H, **p<0.01).

Fig. 4. Effect of acute hypoxia on IKv in PASMCs from control and 2d-H rats. (A, B) Representative current traces obtained by step pulses (between $-80$ mV and 20 mV, 20 mV interval, 500 ms duration) from $-60$ mV of holding potential. The recording was done at 37°C. Acute hypoxia (P O$_2$ 3%) decreased the amplitudes of IKv in PASMCs similarly between control and 2d-H PASMCs. (C) Summary of outward current amplitudes at 0 mV normalized to each control (Ctrl-rats; control, H; hypoxia, *p<0.05). The amplitudes of outward currents were measured at the end of step pulses as indicated by downward arrows above (A, B).

Next, we analyzed whether the hypoxic inhibition of IKv is affected in PASMCs from 2d-H rats. IKv currents were measured in PASMCs using nystatin-perforated whole-cell clamp technique. Application of acute hypoxia (3% P O$_2$) significantly decreased the amplitudes of outward K$^+$ currents (I$_{\text{Kv}}$) in the PASMCs from control and from those from 2d-H rats (Fig. 4, n=5; control, n=4; 2d-H rats).

We have shown previously that in addition to the hypoxic inhibition of IKv, I$_{\text{ISc}}$ activation by U46619 and hypoxia is critical for inducing membrane depolarization in HPV [5]. Therefore, we assessed the I$_{\text{ISc}}$ activation by combined application of U46619 and hypoxia in PASMCs. To record I$_{\text{ISc}}$ selectively, Cs$^+$-rich pipette solution was used to block the voltage-dependent K$^+$ conductance. Application of 50 nM U46619 slightly increased I$_{\text{ISc}}$ at $-60$ mV, which was significantly augmented by combined application of hypoxia with U46619. In contrast, U46619 and hypoxia did not increase I$_{\text{ISc}}$ in PASMCs isolated from 2d-H rats (Fig 5A, B, n=6, respectively).

Fig. 5. Effect of acute hypoxia on I$_{\text{ISc}}$ in PASMCs of control and 2d-H rats. Representative I-V curves of I$_{\text{ISc}}$ were obtained by ramp pulses ($-80$ mV~$-80$ mV) with CsCl pipette solution. (A) Application of U46619 alone did not significantly increased I$_{\text{ISc}}$, while the additional acute hypoxia significantly increased the conductance. (B) In contrast, I$_{\text{ISc}}$ was not increased by acute hypoxia in 2d-H rats. Summaries of inward current amplitudes at $-60$ mV normalized to each control (U; U46619, H; hypoxia) are display as bar graphs in the right panels (**p<0.01).

DISCUSSION

The present study demonstrates that HPV was almost disappeared in rats exposed to hypoxic condition only for two days. The loss of HPV at 2d-H was observed without structural and morphological changes and was not recovered by Kv inhibitors in VIP lungs. Since previous studies supported that an abnormal proliferation of PASMCs accompanied by electrophysiological changes resulted in vascular remodeling under prolonged hypoxic conditions, the period over two weeks was mostly used for exposure time of hypoxia. [6-8,12,13]. The loss of HPV in CH (>two weeks) rats has been consistently observed by many re-
searchers [6-8,14], however, the attenuated HPV from animals exposed relatively short period has been described in only two studies without elucidating the underlying mechanism [8,10].

In the present study, we clearly demonstrate disappearance of HPV at 2d-H prior to inducing noticeable morphological changes. Considering that PA was severely thickened after 2 weeks of hypoxia (Fig. 2C), the regional blood flow in PAs would be obliterated by only a little further contraction. Even if there was no hypertrophic changes in PAs, persistent and general HPV with ambient hypoxia would induce excessive increase of afterload which may lead to the failure in right ventricle [15]. Consequently, the loss of HPV might be regarded as a survival mechanism allowing sufficient alveolar perfusion under the chronic hypoxic environments (e.g., high altitude conditions).

Hypoxic inhibition of \( I_{\text{SC}} \) in PASMCs is well established to be the mechanism of HPV [2,3,11]. However, the \( K_v \) inhibition would not be the sole mechanism for HPV. Otherwise, pharmacological inhibition of \( K_v \) would have totally mimicked HPV. However, even larger HPV (\( \Delta P_{\text{PAHypox}} \)) was observed with 4-AP pretreatment in the V/P lungs from control rats (Fig. 3A, C). In addition, application of 4-AP did not reverse the loss of HPV in 2d-H rats (Fig. 3B, C). Furthermore, in PASMCs from both control and 2d-H rats, the hypoxic inhibition of \( I_{\text{SC}} \) was similar (Fig. 4). These results suggest that apart from \( K_v \) inhibition, there may be another depolarizing mechanism underlying the impaired HPV.

Our previous studies demonstrated that thromboxane \( A_2 \) was required to initiate HPV in isometric tension measurement of isolated PAs and precision-cut lung slice from rats [5,16]. As for the underlying mechanism of the necessity of \( \text{TXA}_2 \), we suggested the hypoxic augmentation of \( I_{\text{SC,TXA2}} \) along with the \( K_v \) inhibition. The most intriguing finding in this study was disappearance of \( \text{O}_2 \)-sensitive \( I_{\text{SC}} \) in PASMCs from 2d-H rats. The functional downregulation of \( I_{\text{SC}} \) might explain the early loss of HPV in 2d-H rats. Further investigation is required to elucidate the signaling mechanisms for the above phenomenon. The molecular nature of \( I_{\text{SC,TXA2}} \) was suggested as TRPC2 according to our previous study [5]. However, other researchers have claimed that TRPC6 channels play critical roles in HPV and idio-pathic pulmonary hypertension [17,20]. In this respect, it would be an interesting direction to pursue the specific types of TRP channel family that is downregulated by chronic hypoxia.

For studying HPV in CH rats, several weeks of continuous or intermittent hypoxia have been conventionally applied in most of the previous researches [7,9,14,21] in CH animal would cause right ventricular hypertrophy and right heart failure [22], which would have clinical implication in chronic obstructive pulmonary disease, sleep apnea and high altitude sickness [6,22-25]. Although no significant medial thickening of PA and right ventricular hypertrophy were observed in 2d-H rats of the present study, slight increase of basal PAP was observed (Fig. 1D). Such results suggested that intrinsic tone of PA was actually increased or unidentified endogenous vasoactive substance might be produced in the lung parenchyme of 2d-H rats. Such an early increase in PA tone would inevitably increase the afterload to right heart, inducing right ventricular hypertrophy in the long term. Mechanisms for the early increase in PA resistance remain to be elucidated.

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REFERENCES

1. Weir EK, Archer SL. The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB J.* 1995;9:183-189.
2. Archer SL, Wu XF, Thébaud B, Nair A, Bonnet S, Tyrrell B, McMurtry MS, Hashimoto K, Harry G, Michelakis ED. Preferential expression and function of voltage-gated, \( \text{O}_2 \)-sensitive \( K_v \) channels in resistance pulmonary arteries explains regional heterogeneity in hypoxic pulmonary vasoconstriction: ionic diversity in smooth muscle cells. *Circ. Res.* 2004;95:308-318.
3. Osipenko ON, Tate RJ, Gurney AM. Potential role for \( K_v3.1 \) channels as oxygen sensors. *Circ. Res.* 2000;86:534-540.
4. Swenson M, Yuan JX. Hypoxic pulmonary vasoconstriction: role of voltage-gated potassium channels. *Respir Res.* 2006;1:40-48.
5. Yoo HY, Park SJ, Jeo SY, Park KS, Han JA, Kim KS, Shin DH, Earm YE, Zhang YH, Kim SJ. Role of thromboxane \( A_2 \) activated nonselective cation channels in hypoxic pulmonary vasoconstriction of rat. *Am J Physiol Cell Physiol.* 2012;302:C307-317.
6. Pierson DJ. Pathophysiology and clinical effects of chronic hypoxia. *Respir Care.* 2000;45:39-51; discussion 51-53.
7. Porez ZL, Michelakis ED, McMurtry MS, Thébaud B, Wu XC, Dyck JR, Hashimoto K, Wang S, Moudgil R, Harry G, Sultanian R, Koshal A, Archer SL. In vivo gene transfer of the \( \text{O}_2 \)-sensitive potassium channel KVL.5 reduces pulmonary hypertension and restores hypoxic pulmonary vasoconstriction in chronically hypoxic rats. *Circulation.* 2003;107:2037-2044.
8. Reeve HL, Michelakis E, Nelson DP, Weir EK, Archer SL. Alterations in a redox oxygen sensing mechanism in chronic hypoxia. *J Appl Physiol (1985).* 2001;90:2249-2256.
9. McMurtry IF, Petrun DO, Reeves JF. Lungs from chronically hypoxic rats have decreased pressor response to acute hypoxia. *Am J Physiol.* 1978;235:H104-109.
10. Asano K, Yanagidaira Y, Yoshimura K, Sakai A. The eGMP pathway is not responsible for the blunted hypoxic vasoconstriction in rat lungs after altitude exposure. *Acta Physiol Scand.* 1997;160:393-400.
11. Moudgil R, Michelakis ED, Archer SL. The role of \( K_v \) channels in determining pulmonary vascular tone, oxygen sensing, cell proliferation, and apoptosis: implications in hypoxic pulmonary vasoconstriction and pulmonary arterial hypertension. *Microcirculation.* 2006;13:615-632.
12. Steenmark KR, Fagan KA, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ Res.* 2006;99:675-691.
13. Laursen BE, Dam MY, Mulvany MJ, Simonsen U. Hypoxia-induced pulmonary vascular remodeling and right ventricular hypertrophy is unaltered by long-term oral L-arginine administration. *J Appl Physiol (1985).* 2006;99:49-55.
14. Osman P, Graham JS, Sweeney M, Yuan JX. Pulmonary vasoconstriction in precision-cut lung slices of rat. *J Physiol Lung Cell Mol Physiol.* 1997;235:H104-109.
15. McMurtry IF, Petrun DO, Reeves JT. Lungs from chronically hypoxic rats have decreased pressor response to acute hypoxia. *Am J Physiol.* 1978;235:H104-109.
16. Park SJ, Yoo HY, Kim HJ, Kim JK, Zhang YH, Kim SJ. Requirement of pretone by thromboxane \( A_2 \) for hypoxic pulmonary vasoconstriction in precision-cut lung slices of rat.
17. Peng G, Lu W, Li X, Chen Y, Zhong N, Ran P, Wang J. Expression of store-operated Ca\(^{2+}\) entry and transient receptor potential canonical and vanilloid-related proteins in rat distal pulmonary venous smooth muscle. *Am J Physiol Lung Cell Mol Physiol.* 2010;299:L621-630.

18. Weissmann N, Dietrich A, Fuchs B, Kalwa H, Ay M, Dumitrascu R, Olschewski A, Storch U, Mederos y Schnitzler M, Ghofrani HA, Schermuly RT, Pinkenburg O, Seeger W, Grimminger F, Guermann T. Classical transient receptor potential channel 6 (TRPC6) is essential for hypoxic pulmonary vasoconstriction and alveolar gas exchange. *Proc Natl Acad Sci U S A.* 2006;103:19093-19098.

19. Yu Y, Fantozzi I, Remillard CV, Landsberg JW, Kunichika N, Platoshyn O, Tigges DD, Thistlethwaite PA, Rubin L, Yuan JX. Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A.* 2004;101:13861-13866.

20. Urban N, Hill K, Wang L, Kuebler WM, Schaefer M. Novel pharmacological TRPC inhibitors block hypoxia-induced vasoconstriction. *Cell Calcium.* 2012;51:194-206.

21. Fagan KA, Oka M, Bauer NR, Gebb SA, Ivy DD, Morris KG, McMurtry IF. Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. *Am J Physiol Lung Cell Mol Physiol.* 2004;287:L656-664.

22. Naeije R, Barberà JA. Pulmonary hypertension associated with COPD. *Crit Care.* 2001;5:286-289.

23. Zubieta-Castillo G Sr, Zubieta-Calleja GR Jr, Zubieta-Calleja L. Chronic mountain sickness: the reaction of physical disorders to chronic hypoxia. *J Physiol Pharmacol.* 2006;57 Suppl 4:431-442.

24. Voelkel NF, Tuder RM. Hypoxia-induced pulmonary vascular remodeling: a model for what human disease? *J Clin Invest.* 2000;106:733-738.

25. McGuire M, Bradford A. Chronic intermittent hypercapnic hypoxia increases pulmonary arterial pressure and haematocrit in rats. *Eur Respir J.* 2001;18:279-285.