Hypoxia-induced pulmonary vasoconstriction of intra-acinar arteries is impaired in NADPH oxidase 4 gene-deficient mice

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Abstract
We show that genetic deficiency of the reactive oxygen species generating enzyme NADPH oxidase 4 (NOX4) impairs hypoxic pulmonary vasoconstriction in small (25–40 μm) intra-acinar, but not pre-acinar, arteries in murine precision cut lung slices. These data suggest an involvement of NOX4 in ventilation-perfusion matching at the acinar level.

Keywords
NADPH oxidase 4 (NOX4), hypoxic pulmonary vasoconstriction, videomorphometry, precision-cut lung slices, NOX4 knockout mice

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Letter
In the short term, hypoxia induces constriction of pulmonary arteries; in the long term, it results in remodeling and narrowing of pulmonary arteries leading to high pulmonary blood pressure and ultimately to cor pulmonale. Barman et al.1 have clearly demonstrated that the reactive oxygen species (ROS; which include hydrogen peroxide, superoxide anion, and hydroxyl radical) generating enzyme NADPH oxidase 4 (NOX4) is expressed in pulmonary arteries and contributes to hypoxia-induced remodeling. In the monocrotaline rat model of pulmonary hypertension (PH), NOX4 inhibitors prevented the development of PH and, furthermore, in a curative setting in which the NOX4 inhibitor VCC202273 was administered to rats with established PH, the progression of disease was attenuated. In view of still missing effective therapies for PH, Barman et al.1 concluded that targeting NOX4 might represent a new therapeutic approach.

However, the impact of NOX4 on the short-term pulmonary responses to hypoxia is unclear. In contrast to other NOX enzymes, NOX4 expressed in cell models shows high constitutive activity in the absence of activators such as phorbol myristate acetate (PMA) or calcium,2 leading to the concept that generation of ROS by NOX4 is mainly regulated at the level of expression. Moreover, Nisimoto et al.3 clearly demonstrated production of oxygen concentration dependent hydrogen peroxide by 293 human embryonic kidney cells stably expressing NOX4, and this production was approximately linear in the range of 1–21% oxygen. Furthermore, Mittal et al.4 described co-expression of NOX4 and Kv1.5, a voltage gated K+ channel, in isolated rat pulmonary artery smooth muscle cells (PASMCs). NOX4-derived elevation of ROS resulted in a reduction of Kv currents leading to membrane depolarization, Ca2+-influx and contraction of cells. These data make NOX4 an interesting candidate as a rapid acting oxygen sensor. Our study aimed to clarify the contribution of NOX4 to pulmonary vasoconstriction under acute hypoxic conditions by comparison of the responses of wild-type (WT) and NOX4 knockout (KO) mice.

Genotyping, as described in Zhang et al.,5 allowed a clear discrimination between WT and NOX4 KO mice (Fig. 1a). Reverse transcriptase polymerase chain reaction (RT-PCR)
Fig. 1. PCR genotyping of WT and NOX4 KO mice and expression of NOX4 mRNA in lungs and videomorphometric analysis of hypoxic pulmonary vasoconstriction (HPV) of intra-pulmonary arteries of WT and NOX4 KO mice. (a, b) Agarose gels: (a) single bands of 580 bp and of 190 bp appeared in WT and NOX4 KO mice, respectively. (b) NOX4 transcripts were detectable in WT lung but not in KO lung samples. β-Actin is used as housekeeping gene control for RT-PCR; positive control is heart sample; ØRT is control without reverse transcriptase; water is control without template. The band of highest intensity in the 100 bp DNA ladder marker is of 500 bp. The expected products size is indicated on the right side of gel electrophoresis image. The solid lines indicate assembled parts of the same gel. (c, d) In precision-cut lung slices, vasoreactivity of cross-sectioned intra-pulmonary arteries was quantified as relative changes of the luminal area against time. (c) Under hypoxic conditions, small intra-acinar arteries of WT and NOX4 KO mice exhibited about 40% and 20% reduction in the luminal area, respectively. HPV of arteries of NOX4 KO mice was significantly reduced as compared to WT animals. (d) Larger pre-acinar arteries of WT and KO mice responded to hypoxia in a comparable manner. *, #, $, §; P ≤ 0.05; **, ##, $$, §§; P ≤ 0.01 (Mann–Whitney U-test); tests of significances were done at the time points indicated by arrows; data are presented as mean ± standard error of the mean; n refers to the number of arteries/number of animals.
data revealed expression of NOX4 mRNA in the lungs of WT but not of KO mice. In contrast, the housekeeping gene β-actin was equally expressed in both mouse strains (Fig. 1b).

The pulmonary trunk branches and extends towards the periphery of the lungs. The proximal arteries marked with abundant elastic fibers are followed by initially muscularized intra-pulmonary arteries. While extending further, the muscular layer becomes incomplete and ultimately arteries become free of smooth muscle actin-immunoreactive cells. Thus, each segment of the intra-pulmonary arterial system should be expected to behave differently.6 There is increasing evidence that the response to hypoxia varies along the progression of the pulmonary vascular tree. For instance, rings of rat pulmonary arteries with lumen diameters of 1.5–2.0 mm displayed a biphasic response to hypoxia.7 Likewise, Tabuchi et al.8 demonstrated prominent monophasic constriction of murine subpleural arterioles having a lumen diameter of 30–50 μm in response to hypoxia, whereas arterioles with a lumen diameter <30 μm showed mild changes in diameter, which were not significant. In isolated mouse pulmonary arteries, hypoxic pulmonary vasoconstriction (HPV) is seen only in small (80–200 μm) but not larger intra-pulmonary vessels.9 We analyzed the role of NOX4 in HPV of intra-pulmonary arteries running in the depth of the lung. For this purpose, cross-sectioned arteries in precision cut lung slices were investigated by videomorphometry; details of this technique are given in Paddenberg et al.10,11 These arteries were subgrouped into small intra-acinar arteries with lumen diameters of 25–40 μm located at the gussets of alveolar septa and surrounded by alveolae and larger pre-acinar arteries with lumen diameters of 41–60 μm running in the close neighborhood to bronchi and bronchioles. In intra-acinar arteries of WT mice, hypoxia induced about 40% reduction of the luminal area indicating vasoconstriction. In intra-acinar arteries of KO animals, HPV was significantly reduced (Fig. 1c). In contrast, the hypoxic response of pre-acinar arteries was comparable in WT and KO mice resulting in both cases in about 20% reduction of the luminal area (Fig. 1d). These differential responses might reflect the presence of different patterns of smooth muscle cell layers and morphologically different contractile cells in both types of arteries.12 In line with our present data, a similar vessel-specimen-specific response to hypoxia was demonstrated by Paddenberg et al.,11 where HPV was sustained in larger pre-acinar arteries and abolished in small intra-acinar arteries from succinate dehydrogenase complex subunit D (SDHD) heterozygous mice. Moreover, pulmonary arterial pressure (PAP) recordings from perfused and hypoxic ventilated lungs were comparable between heterozygous and WT mice. Differential reactivity of vessels was ascribed to diversity of contractile cells and their sensitivity to hormones and neurotransmitters as well as their reactivity to hypoxia.

Previous studies have presented evidence for an increase in NOX4 expression in cultured human and rat PASMCs under hypoxic conditions, in lungs of hypoxia-induced PH animal models, and in lungs of patients having idiopathic pulmonary arterial hypertension.1,4,13-15 Moreover, NOX4 is involved in cell proliferation and ROS production,4 as its dysfunctioning/silencing results in impairment of ROS formation and PASM C proliferation in human16 and rat.4 Thus, the hypothesis may arise that under hypoxic conditions, an increase in NOX4 expression results in increased ROS formation contributing to HPV. However, in a recent study,17 hypoxia-induced PAP in isolated, perfused, and ventilated lungs, elevation of right ventricular systolic pressure, increase in right heart hypertrophy, and degree of remodeling of fully, partially, and non-muscularized pulmonary vessels remained unimpaired in NOX4 KO mice. In line with these data, we did not observe impairment in the HPV of pre-acinar arteries in the same KO mice. Thus, strong evidence is provided for the non-involvement of NOX4-derived ROS in hypoxia-induced PH in mice.17

Still, we observed impairment of HPV of NOX4-KO intra-acinar arteries, suggesting that this vascular segment does not significantly contribute to overall PAP. This conclusion is further supported by previous data obtained in two other gene-deficient mouse strains. Similar to NOX4 KO mice, heterozygous SDHD-deficient mice show impaired HPV of intra-acinar arteries, but intact hypoxia-induced rise in PAP.11 Conversely, transient receptor potential cation channel subfamily C member 6 (TRPC6) KO mice present intact HPV of small intrapulmonary vessels9 but lack an acute PAP response to hypoxia.18 In case of SDHD heterozygous mice, we previously showed that this impairment of intra-acinar HPV was associated with disturbed ventilation/perfusion matching.11

In conclusion, our data in conjunction with a previous study17 demonstrate that NOX4 contributes to neither hypoxia-induced rise in PAP nor HPV of larger pre-acinar arteries. However, NOX4 plays a marked role in HPV of intra-acinar arteries, which are linked to ventilation-perfusion matching. Thus, targeting NOX4 as a new therapeutic approach, as suggested to suppress pulmonary arterial remodeling,1 may in addition to the expected beneficial attenuation of progression of PH have an adverse impact on short-term ventilation-perfusion matching.

For details to the methods used in this study, see Electronic Supplementary Material. All experiments in this study were approved by the Regierungspräsidium Giessen, Germany (approval No. G1 20/23 A7/2009).

Conflict of interest
The author(s) declare that there is no conflict of interest.

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References

1. Barman SA, Chen F, Su Y, et al. NADPH oxidase 4 is expressed in pulmonary artery adventitia and contributes to hypertensive vascular remodeling. *Arterioscler Thromb Vasc Biol* 2014; 34(8): 1704–1715.

2. Lambeth JD. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004; 4(3): 181–189.

3. Nisimoto Y, Diebold BA, Cosentino-Gomes D, et al. Nox4: a hydrogen peroxide-generating oxygen sensor. *Biochemistry* 2014; 53(31): 5111–5120.

4. Mittal M, Roth M, Konig P, et al. Hypoxia-dependent regulation of nonphagocytic NADPH oxidase subunit NOX4 in the pulmonary vasculature. *Circ Res* 2007; 101(3): 258–267.

5. Zhang M, Brewer AC, Schröder K, et al. NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. *Proc Natl Acad Sci USA* 2010; 107(42): 18121–18126.

6. Drexler ES, Quinn TP, Slifka AJ, et al. Comparison of mechanical behavior among the extrapulmonary arteries from rats. *J Biomech* 2007; 40(4): 812–819.

7. Bennie RE, Packer CS, Powell DR, et al. Biphasic contractile response of pulmonary artery to hypoxia. *Am J Physiol* 1991; 261(2 Pt 1): L156–163.

8. Tabuchi A, Mertens M, Kuppe H, et al. Intravital microscopy of the murine pulmonary microcirculation. *J Appl Physiol (1985)* 2008; 104(2): 338–346.

9. Strielkov I, Krause NC, Sommer N, et al. Hypoxic pulmonary vasoconstriction in isolated mouse pulmonary arterial vessels. *Exp Physiol* 2018; 103: 1185–1191.

10. Paddenberg R, Mermer P, Goldenberg A, et al. Videomorphometric analysis of hypoxic pulmonary vasoconstriction of intra-pulmonary arteries using murine precision cut lung slices. *J Vis Exp* 2014; 83): e50970.

11. Paddenberg R, Tiefenbach M, Faulhammer P, et al. Mitochondrial complex II is essential for hypoxia-induced pulmonary vasoconstriction of intra- but not of pre-acinar arteries. *Cardiovasc Res* 2012; 93(4): 702–710.

12. Davies P, Burke G and Reid L. The structure of the wall of the rat intraacinar pulmonary artery: an electron microscopic study of microdissected preparations. *Microvasc Res* 1986; 32(1): 50–63.

13. Adesina SE, Kang BY, Bijli KM, et al. Targeting mitochondrial reactive oxygen species to modulate hypoxia-induced pulmonary hypertension. *Free Radic Biol Med* 2015; 87: 36–47.

14. Lu X, Murphy TC, Nanes MS, et al. PPAR{gamma} regulates hypoxia-induced Nox4 expression in human pulmonary artery smooth muscle cells through NF-κB. *Am J Physiol Lung Cell Mol Physiol* 2010; 299(4): L559–566.

15. Mittal M, Gu XQ, Pak O, et al. Hypoxia induces Kv channel current inhibition by increased NADPH oxidase-derived reactive oxygen species. *Free Radic Biol Med* 2012; 52(6): 1033–1042.

16. Ismail S, Sturrock A, Wu P, et al. NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine production of transforming growth factor-β1 and insulin-like growth factor binding protein-3. *Am J Physiol Lung Cell Mol Physiol* 2009; 296(3): L489–499.

17. Veith C, Kraut S, Wilhelm J, et al. NADPH oxidase 4 is not involved in hypoxia-induced pulmonary hypertension. *Pulm Circ* 2016; 6(3): 397–400.

18. Weissmann N, Dietrich A, Fuchs B, et al. 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(50): 19093–19098.

19. Brandes RP, Weissmann N and Schröder K. NADPH oxidases in cardiovascular disease. *Free Radic Biol Med* 2010; 49(5): 687–706.