SHORT COMMUNICATION

Hypoxia-Inducible Factors Activator, Roxadustat, Increases Pulmonary Vascular Resistance in Rats

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Summary

Activators of hypoxia inducible factors (HIFs), such as roxadustat, are promising agents for anemia treatment. However, since HIFs are also involved in the regulation of the pulmonary circulation, we hypothesized that roxadustat increases pulmonary vascular resistance and vasoconstrictor reactivity. Using isolated, cell-free solution perfused rat lungs, we found perfusion pressure-flow curves to be shifted to higher pressures by 2 weeks of roxadustat treatment (10 mg/kg every other day), although not as much as by chronic hypoxic exposure. Vasoconstrictor reactivity to angiotensin II and acute hypoxic challenges was not altered by roxadustat. Since roxadustat may inhibit angiotensin-converting enzyme 2 (ACE2), we also tested a purported ACE2 activator, diminazene aceturate (DIZE, 0.1 mM). It produced paradoxical, unexplained pulmonary vasoconstriction. We conclude that the risk of serious pulmonary hypertension is not high when roxadustat is given for 14 days, but monitoring is advisable.

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Expression of a large number of genes is adjusted to oxygen availability through the hypoxia-inducible factor (HIF) family of transcription factors [1].

HIFs are heterodimers. While the β subunit is stable, the α subunit is labeled by ubiquitination in the presence of oxygen and degraded. Thus, the dimer cannot form and the expression of genes controlled by HIFs does not occur. When oxygen is deficient, ubiquitination is limited, the α subunit survives, forms a dimer with the β subunit, and transcription of corresponding genes ensues. The enzyme that utilizes oxygen to initiate ubiquitination of the α subunit is prolyl hydroxylase [1]. Pharmacological inhibition of this enzyme in normoxia therefore acts on the HIF system similarly to hypoxia, limiting the degradation of the α-subunit and thus allowing the formation of a complete HIF dimer.

As the first gene for which HIF regulation was described was erythropoietin [1], pharmacological interference with the HIF system has been investigated as a possible therapy for anemia. Of particular interest is a group of prolyl hydroxylase inhibitors (e.g. roxadustat) [2]. While especially the possibility to use them orally is attractive, there is still a controversy regarding their safety, especially cardiovascular (e.g. roxadustat has been approved by EMA, but not FDA).

One of the systems significantly affected by hypoxia is the pulmonary circulation. Chronic hypoxia is an important factor in the development of pulmonary hypertension, a disorder associated with significant morbidity and mortality [3]. There is evidence that HIF activation is part of its mechanism [4]. Thus, a risk of pulmonary hypertension might be a potential concern when using drugs such as roxadustat. This is indirectly

supported by the finding of pulmonary hypertension in endothelial prolyl hydroxylase 2 gene knockout mice [5].

Roxadustat seems to have other effects in addition to HIF stabilization. For example, it can downregulate angiotensin-converting enzyme 2 (ACE2) activity [2]. ACE2 acts against pulmonary hypertension [6]. ACE2 inhibition thus could be an additional mechanism whereby roxadustat might promote pulmonary hypertension.

The aim of this study therefore was to evaluate the effect of an HIF activator on the development of pulmonary hypertension and to assess the involvement of ACE2 in this process.

The experiments were performed using adult male Wistar rats (AnLab, Prague, Czech Republic) in accordance with the European Guidelines on Laboratory Animal Care. They were approved by the Ministry of Education, Youth and Sports as the Czech Republic's authority competent to approve experimental projects pursuant to the Animal Protection Act (#AVCR 4304/2021 SOV I). Rats were kept at room temperature with free access to standard diet (AnLab) and tap water.

One group was treated with roxadustat for 2 weeks (group Rox2w, n=9). Five milligrams of roxadustat (MedChemExpress, Monmouth Junction, NJ, USA; all other drugs were from Sigma-Aldrich, Prague, Czech Republic) were dissolved in 25 µl DMSO + 200 µl PEG + 25 µl TWEEN 80+250 µl water and injected i.p. (10 mg/kg BW) every other day [2,7]. Since we expected that it will increase hematocrit, which itself elevates pulmonary vascular resistance, we used another group that received roxadustat only once and was studied 48 h later, so that pulmonary vascular mechanisms controlled by HIF could be altered, but the effect on hematocrit has not yet manifested (Rox2d, n=9). A control group was treated the same way as Rox2w, except that they were injected with solvent without roxadustat (n=8). To check for possible effects of the solvent itself, a group was included that was not treated in any way (untreated group, n=5). To compare the results with a situation of naturally elevated HIFs, the last group was treated identically to controls, except that it was exposed to chronic hypoxia (10 % O_2) for the two weeks of solvent injections (group chronic hypoxia CH, n=6).

The pulmonary vasoresistive properties were evaluated by analyzing the perfusion pressure-flow relationship in isolated lungs perfused *ex vivo* at 40 ml/min/kg BW with a cell-free Krebs solution with 4 % albumin (to exclude the confounding effect of hematocrit variations) and ventilated with air + 5 % CO_2 at 60 breaths/min with a +2 cm H_2O end-expiratory pressure [8,9]. Before lung isolation, the rats were anesthetized with thiopental (30 mg/kg BW i.p.) and a blood sample was taken by cardiac puncture to measure hematocrit. The pressure-flow relationship was determined by measuring the pressure while the perfusion was first stopped for ~30 s and then increased to 36 ml/min/kg BW in 3 even steps (~30 s each). Since the pressure-flow relationship in this range appeared close to linear, it was evaluated with linear regression (R^2>0.8925).

Vasoconstrictor reactivity was then tested by our routine sequence of injecting angiotensin II (0.15 µg) into the inflow line, followed, after perfusion pressure stabilization (~8 min), with ventilatory hypoxia (0 % O_2 + 5 % CO_2, 10 min). This angiotensin II + acute hypoxia sequence was repeated once more. To test whether the pulmonary vascular resistive properties and/or vasoconstrictor reactivity were altered by the possible inhibitory effect of roxadustat on ACE2 [2], a putative ACE2 stimulator, diminazene aceturate (DIZE) [10], was then added to the perfusate (0.1 mM). After a 10-min stabilization, the whole protocol (pressure-flow measurement, angiotensin II plus acute hypoxia twice) was repeated (Fig. 1).

The results were analyzed statistically with Prism 9.5 software (GraphPad Software, Boston, MA, USA) and presented as means ± SD. Groups were compared using one-way ANOVA with Fisher's Least significant difference test. The pressure-flow curves before and after DIZE administration were evaluated separately in each group using two-way repeated measures ANOVA. The vasoconstrictor responses before and after DIZE were compared separately for each group with paired *t*-test. *P*<0.05 was considered significant.

Chronic but not acute roxadustat treatment increased hematocrit compared both to control and untreated group, but not as much as chronic hypoxia (Table 1).

Baseline perfusion pressure in the isolated lungs was higher in the Rox2d group (9.8±1.6 mm Hg) than in controls (7.9±1.8, *P*=0.0364). It was even higher in the CH group (13.0±2.0), compared both to controls and untreated (*P*<0.0001) and to both roxadustat groups (*P*≤0.0011). It was 9.2±1.9 in the Rox2w and 8.1±1.2 mm Hg in the untreated rats.
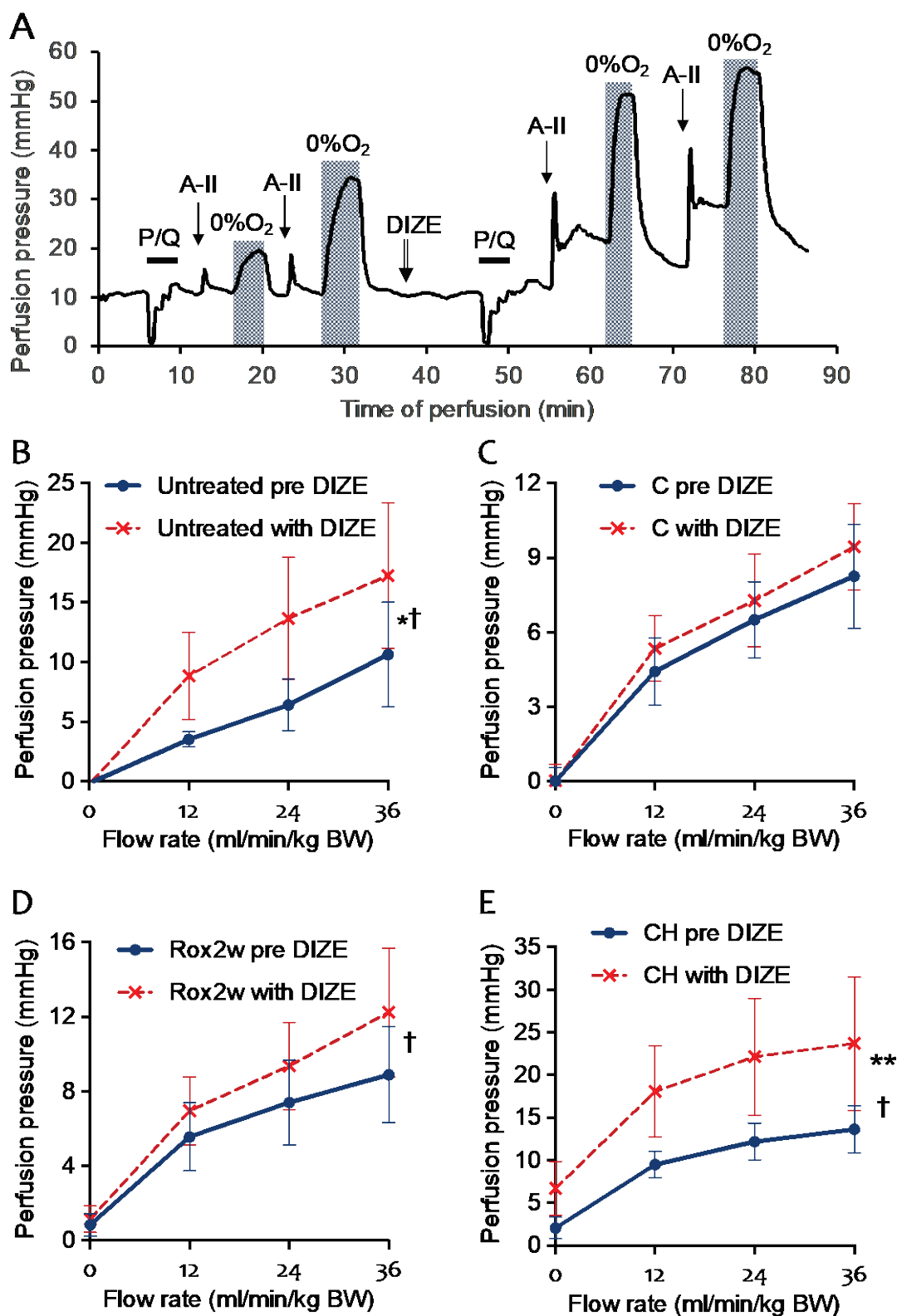
The slope of the pressure-flow regression lines

Fig. 1. (**A**) Example pressure recording (Rox2w group) illustrating the lung perfusion protocol. P/Q, pressure-flow curve measurement; A-II, inflow injection of 0.15 µg angiotensin II; 0 % O_2, hypoxic challenge; DIZE, 0.1 mM diminazene aceturate added to the perfusate. (**B-E**) Perfusion pressure-flow curves are shifted to higher pressures by diminazene aceturate (DIZE) in untreated (B, n=5), roxadustat-treated (for 2 weeks, Rox2w, n=7; panel D) and chronically hypoxic (CH, n=6; E), but not control (C, n=7, panel C) and roxadustat-treated (once before the measurement, Rox2d, n=7, not shown) groups. Flow was a significant source of variation in all groups ($P<0.0001$, two-way repeated measures ANOVA). * $P=0.0453$ effect of group (values after DIZE are higher than values before DIZE even when flow is not considered). ** $P=0.0064$ effect of group (pre vs. post DIZE). † $P≤0.0055$ effect of group (pre vs. post DIZE) × flow interaction (values after DIZE are getting more different from the values before DIZE as flow increases).

Table 1. Hematocrit and vasoconstrictor reactivity (second repetition of the challenges).

Group	Hematocrit (%)	Pressure-flow line intercept with the pressure axis (mm Hg)	Angiotensin II (Δ perfusion pressure, mm Hg)	Acute 0 % O_2 (Δ perfusion pressure, mm Hg)
Untreated (n=5)	39.6±2.3	1.5±0.8	9.8±5.1	23.8±9.8
Control (n=8)	39.7±2.5	2.6±1.4	10.8±5.6	23.3±12.5
Rox2d (n=9)	41.8±2.3	4.2±1.5*,†	7.4±2.3	17.1±7.4
Rox2w (n=9)	46.6±1.8**,††,‡‡	3.9±1.5*,†	12.8±6.0‡	26.5±11.4
CH (n=6)	49.8±1.7**,†††,‡‡,§	7.6±1.1**,†††,‡‡,§§	20.9±5.7**,††,‡‡,§§	28.6±8.6

* $P<0.05$ vs. Control, ** $P\leq0.0008$ vs. Control, † $P<0.01$ vs. Untreated, †† $P<0.001$ vs. Untreated, ††† $P<0.0001$ vs. Untreated, ‡ $P=0.0284$ vs. Rox2d, ‡‡ $P\leq0.0004$ vs. Rox2d, § $P<0.05$ vs. Rox2w, §§ $P\leq0.0049$ vs. Rox2w.

did not differ among the groups (i.e. the lines were parallel), whereas the intercept with the pressure axis was increased by roxadustat already after 2 days and did not further change after 2 weeks compared to control and untreated groups (that did not differ one from another, Table 1). The pressure axis intercept was even higher in the CH group. This parameter corresponds to the critical closing pressure of the pulmonary vasculature [11].

Consistent with this, the value at which the perfusion pressure equilibrated when perfusion was stopped did not differ between control (0.1±0.6 mm Hg) and untreated (-0.2±0.5, $P=0.6737$) groups, but was elevated similarly ($P<0.05$) in the Rox2w (0.8±0.4) and Rox2w (0.8±0.6) groups and even more so in the CH group (2.1±1.3, $P\leq0.0028$). The stop-flow pressure reflects critical closing pressure (if critical closing pressure was zero, then the pressure on the inflow side should equilibrate with the outflow pressure of -2 cm H_2O).

The vasoconstrictor reactivity to angiotensin II was potentiated by chronic hypoxia, as reported previously [12], but was not altered by Roxadustat compared to control or untreated rats. It was higher in the Rox2w than Rox2d group (Table 1). Reactivity to acute hypoxic challenges did not differ among the groups.

DIZE did not alter the pressure-flow lines in the control and Rox2w groups, but it shifted them to higher pressures in the untreated, Rox2w, and CH groups (Fig. 1B-E). The vasoconstrictor responses to acute

hypoxia were not altered, while those to angiotensin II rose after DIZE in controls (to 18.2±6.8 mm Hg, $P=0.0148$ vs. before DIZE), Rox2d (11.5±6.2, $P=0.0387$) and Rox2w (17.8±6.1, $P=0.0004$) groups, but not in the CH group (19.6±6.2, $P=0.3171$).

These data show that inhibition of prolyl hydroxylases (and thus HIF activation) by roxadustat elevates pulmonary vascular critical closing pressure, thus shifting pressures over a range of flow rates to higher values. This effect is independent of the influence of roxadustat on hematocrit, but is smaller than the established pulmonary hypertension model of chronic hypoxia. In line with our previous observations that essential changes in the development of chronic hypoxic pulmonary hypertension occur during the first days of hypoxic exposure [13], the effect of roxadustat on the pulmonary circulation is faster (already after 2 days) than on hematocrit. Since we detected the effect of roxadustat in lungs perfused with a cell-free solution, it is likely that *in vivo*, where the positive effect of roxadustat on hematocrit is also present, its effect on pulmonary vascular resistance will be even greater.

Acute pulmonary vasoconstrictor reactivity (to angiotensin II and 0 % O_2) was not affected by roxadustat. This is surprising, inasmuch roxadustat was reported to downregulate vascular AT_1 receptors [14]. Furthermore, the expression and activity of voltage-gated K^+ channels, important in the mechanism of acute hypoxic vasoconstriction [15], are affected by HIFs [4].

For example, both chronic hypoxia and HIF-1 over-expression in normoxia reduced the expression of Kv1.5 and Kv2.1 channels in intrapulmonary arterial smooth muscle cells *via* endothelin-1 upregulation [16]. Nevertheless, our data suggest that this influence is not functionally very powerful.

Antitrypanosomal drug and ACE2 stimulator DIZE [10] was reported to stop the progression of experimental pulmonary hypertension [17]. Our findings of the pressure-flow lines shifted to higher pressures and potentiated angiotensin II reactivity in most of our experimental groups is thus surprising. It is likely that some DIZE actions other than ACE2 activation played a role. One possibility could be DIZE's interference with acid-sensitive ion channels [18], recently shown to be important in pulmonary vascular smooth muscle regulation [19]. Although we do not understand this paradoxical effect of DIZE, we consider useful reporting it, especially as it is commonly used as an antiparasitic.

In conclusion, the risk of HIF stabilizer-induced pulmonary hypertension is real and worth considering, especially as pulmonary hypertension is a common complication of chronic kidney disease even without such treatment [20]. Fortunately, increased pulmonary vasoconstrictor reactivity, which would be an additional risk factor, does not occur with roxadustat. HIFs do not seem to play a significant role in hypoxic pulmonary vasoconstriction. ACE2 stimulator, DIZE, may cause paradoxical pulmonary vasoconstriction.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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