SuHx rat model: partly reversible pulmonary hypertension and progressive intima obstruction

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ABSTRACT The SU5416 combined with hypoxia (SuHx) rat model features angio-obliterative pulmonary hypertension resembling human pulmonary arterial hypertension. Despite increasing use of this model, a comprehensive haemodynamic characterisation in conscious rats has not been reported.

We used telemetry to characterise haemodynamic responses in SuHx rats and associated these with serial histology.

Right ventricular systolic pressure (RVSP) increased to a mean $\pm$ SD of 106 $\pm$ 7 mmHg in response to SuHx and decreased but remained elevated at 72 $\pm$ 8 mmHg upon return to normoxia. Hypoxia-only exposed rats showed a similar initial increase in RVSP, a lower maximum RVSP and near-normalisation of RVSP during subsequent normoxia. Progressive vascular remodelling consisted of a four-fold increase in intima thickness, while only minimal changes in media thickness were found. The circadian range in RVSP provided an accurate longitudinal estimate of vascular remodelling.

In conclusion, in SuHx rats, re-exposure to normoxia leads to a partial decrease in pulmonary artery pressure, with persisting hypertension and pulmonary vascular remodelling characterised by progressive intima obstruction.

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Introduction

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease characterised by remodelling of the lung vessels, increased pulmonary vascular resistance and, ultimately, dysfunction of the right ventricle [1]. Animal models of pulmonary hypertension have provided critical insights that help to explain the pathobiology of the disease and have served as a platform for drug development [2]. Traditionally, two animal models have been popular for preclinical testing of new PAH pharmacotherapies: the chronic hypoxia-induced pulmonary hypertension model and the monocrotaline lung injury model [2]. More recently, new animal models based on vascular endothelial growth factor receptor (VEGF-R) blockade with the tyrosine kinase inhibitor SU5416 have been introduced and the SU5416 plus chronic hypoxia (SuHx) model is now being extensively used [3, 4]. Indeed, several investigators have demonstrated that the SuHx model represents many salient features of human PAH, such as severe pulmonary hypertension obliterative vascular lesions, right ventricular dysfunction, decreased exercise endurance and treatment refractoriness [5–7]. Plexiform-like lesions emerge in the pulmonary vasculature of SuHx rats when the normoxic re-exposure protocol is extended to $\geqslant$10 weeks [4, 8]. Whereas histological changes in the lungs and right ventricle of SuHx rats have been described in much detail [8–10], a haemodynamic characterisation of the model has been restricted to right ventricular catheterisations in groups of anesthetised rats at different time-points [11]. Longitudinal haemodynamic measurements in conscious rats, as can be obtained using telemetry, are important to provide a context for the histological changes and to ascertain the value of the model for preclinical drug testing. Therefore, the aim of this study was to characterise the haemodynamic responses in conscious SuHx rats and to associate these with morphological data obtained by serial histology. We show the feasibility of a longitudinal characterisation of the haemodynamic changes in conscious and freely moving SuHx rats and rats exposed to hypoxia alone, by measuring right ventricular systolic pressure (RVSP) nearly continuously by means of an implanted telemetry device. Our study reveals a previously unappreciated but only partial reversibility of pulmonary hypertension in SuHx rats, which may interfere with the interpretation of preclinical drug studies. Despite this partial reversibility in pulmonary hypertension, serial histology showed a progressive remodelling of the pulmonary vascular wall, in particular the intima layer.

Material and methods

Animal model

10 male Sprague Dawley rats (Crl:CD(SD); Charles River, Sulzfeld, Germany) underwent implantation of a telemetric pressure sensor in the right ventricle as described previously [12] (see the online supplementary material for the implantation protocol). Five rats were exposed to hypoxia only (Hx) and five rats to SuHx. Eight additional groups of four SuHx rats were used for serial measurements of right ventricular hypertrophy, histology and haematocrit at baseline and after every subsequent week. SuHx-mediated pulmonary hypertension was induced according to the protocol published previously [4, 13]. SU5416 (Tocris Bioscience, Bristol, UK) was administered to rats weighing $<$200 g as a single subcutaneous injection (25 mg·kg^{-1}) [14] (see the online supplementary material for preparation details) 5–7 days after sensor implantation. SuHx (at day of injection) and Hx rats were housed for 4 weeks in 10% oxygen (Biospherix Ltd, New York, NY, USA) maintained by a nitrogen generator (Avilo, Dirksland, the Netherlands) and subsequently re-exposed to normoxia for 3 weeks. The study was approved by the local animal welfare committee (VU-Fys 11–16).

Telemetry

Telemetry data acquisition consisted of averaged recordings of 1-min duration recorded every hour. The analysis was based on the RVSP at 22:00 h at baseline and at the end of every subsequent week. At the same time-points, the circadian range in the RVSP (ΔRVSP$_{max\text{-}min}$) was determined. In addition, acute changes in RVSP upon a 4-min exposure to hyperoxia were recorded weekly. See the online supplementary material for detailed information.

Echocardiography

To measure right ventricular end-diastolic diameter (RVEDD) and tricuspid annular plane systolic excursion (TAPSE), animals underwent weekly echocardiographic assessments (Prosound SSD-4000 and UST-5542; Aloka, Tokyo, Japan), as published previously [12].

Histological and morphometric analyses

For comparison with weekly telemetry data, necropsy was performed in separate groups of four SuHx rats at baseline and after every week. Lung tissue was processed for histology (online supplementary material). Small pulmonary arteries were divided into three classes, based on external diameters: $<$30, 30–60 and

60–100 µm [15] (see online supplementary material for rationale of vessel classes). Media and intima wall thickness were measured and recorded as described previously [4, 9, 16] and as described in the supplement.

Statistical analyses

Parametric variables were compared between groups using appropriate ANOVA with Bonferroni *post hoc* tests. Correlations were determined using Pearson correlation tests (Graphpad, La Jolla, CA, USA). Data are presented as mean±SD.

Results

Telemetric RVSP and right ventricular hypertrophy measurements

Telemetry was feasible for the measurement of RVSP in conscious rats up to ⩾8 weeks (fig. 1). The RVSP remained constant in a control rat, which confirms the accuracy of the telemetric method. There was no peri-operative or post-operative mortality. However, recordings in one Hx rat did not meet quality criteria and were therefore excluded from the analyses. The RVSP increased upon exposure to hypoxia in Hx and SuHx rats, and both groups showed a comparable rise during the first 2 weeks of hypoxia (fig. 2). Subsequently, SuHx rats showed a trend toward a progressively greater increase in RVSP to values >100 mmHg, while Hx rats showed an increase to maximally ~80 mmHg. Immediately upon re-exposure to normoxia, a rapid 20% decrease in RVSP was observed (fig. 2b). Hx rats showed a significant but less pronounced decrease in RVSP (fig. 2c). After this decrease, a mild reduction of pressure followed in both groups, and at 6 and 7 weeks, RVSP stabilised. At stabilisation, RVSP in the SuHx rat remained elevated and significantly than in Hx rats. Echocardiography in SuHx rats showed an increase in RVEDD and decrease in TAPSE during the hypoxic period, followed by a partial recovery during in the first 2 weeks of normoxic re-exposure and a subsequent further worsening in the last week of the protocol (fig. 2d). RVEDD was temporarily increased in Hx rats, whereas TAPSE was unaffected by hypoxia (fig. 2e). In Hx and SuHx rats alike, brief exposure to hyperoxic conditions revealed an acute reversibility in pressure (fig. 3). This finding was confirmed by catheterisations in the non-telemetry animals (histology groups) during termination, which showed lower RVSP values than those obtained in telemetry rats (online supplementary fig. 4). The acute decrease in pressure equalled the pressure increase over the first week. With continuous re-exposure to normoxia, this vasoreactive response to hyperoxia disappeared. Right ventricular hypertrophy (expressed as right ventricular weight over left ventricular plus septal weight (RV/(LV+S))) in the SuHx group increased progressively during hypoxia and partially decreased upon normoxic re-exposure (fig. 4a). The RV/(LV+S) correlated with the RVSP throughout the entire study period (fig. 4b). The haematocrit in SuHx rats increased significantly during the hypoxic period and normalised within 1 week after re-exposure to normoxic conditions (fig. 4c). Haematocrit correlated with RVSP during the hypoxic period only (fig. 4d).

Circadian range of RVSP

During hypoxic exposure, both groups showed a progressive increase in $\Delta RVSP_{max\text{-}min}$, while upon normoxic re-exposure, the $\Delta RVSP_{max\text{-}min}$ decreased in Hx rats but continued to increase in SuHx rats (fig. 5).

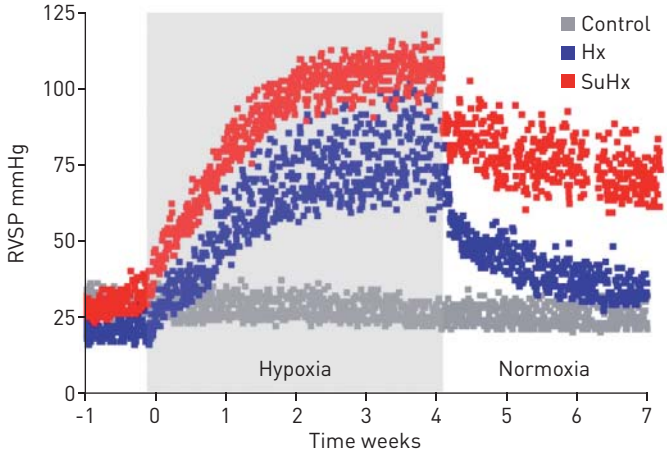

FIGURE 1 All pressure recordings (1-min average for every hour of acquisition) in one representative rat exposed to hypoxia only (Hx) and one rat exposed to SU5416 and hypoxia (SuHx). Every square represents 1 h of data acquisition. Data of one control rat (normal range of right ventricular systolic pressure (RVSP)) is also shown.

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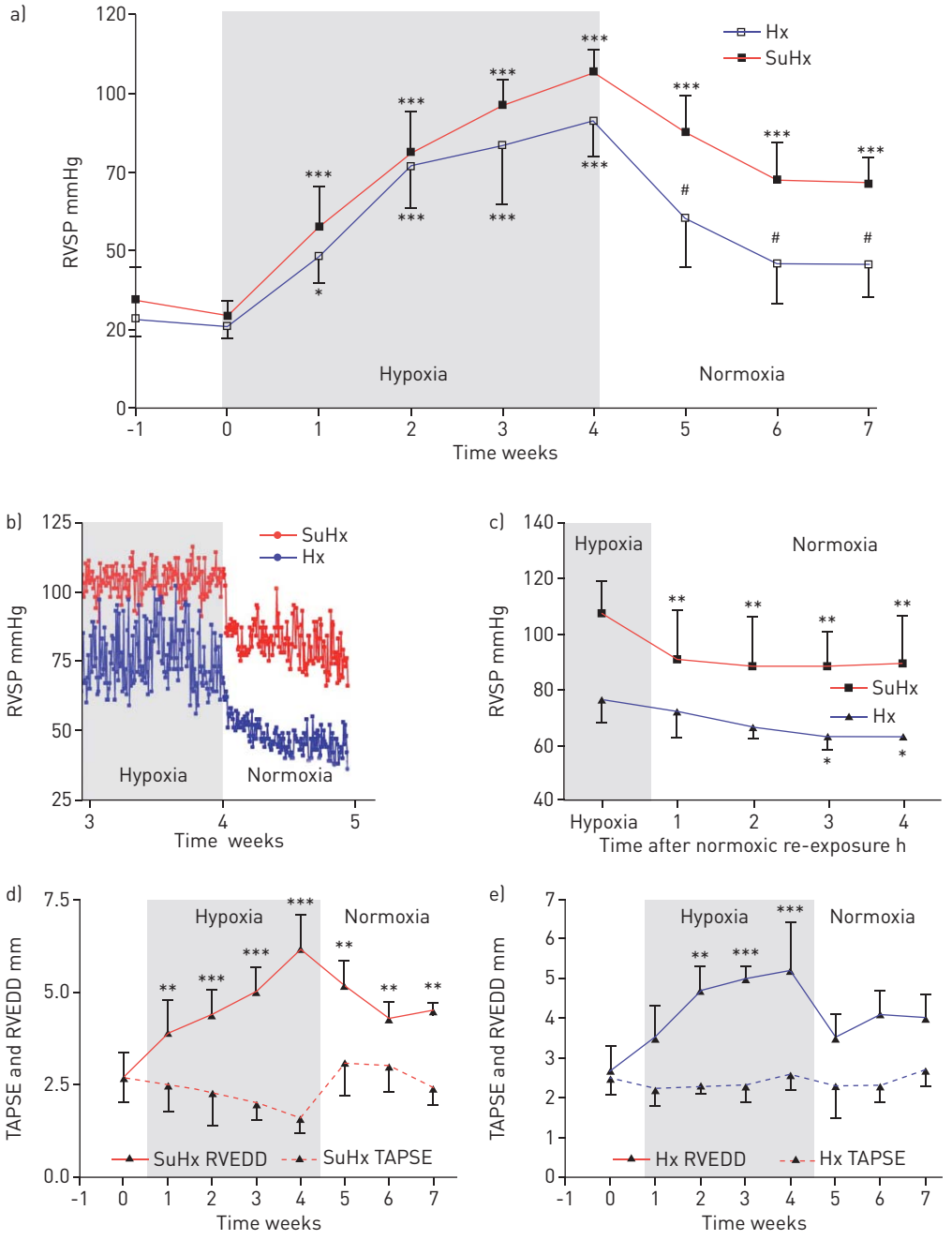

FIGURE 2 a) Mean right ventricular systolic pressure (RVSP) throughout the entire study period in rats exposed to hypoxia only (Hx) (n=4) and exposed to SU5416 and hypoxia (SuHx) (n=5). *: p<0.05 *versus* baseline; ***: p<0.001 *versus* baseline; #: p<0.05 *versus* Hx. b) Re-exposure to normoxia results in an acute 20% decrease in RVSP within 1 h in a representative SuHx animal (data points each hour). c) To visualise the acute decrease in RVSP upon normoxic re-exposure, significant differences between the mean RVSP measured during the last day of hypoxia and the first four normoxia time-points in SuHx and Hx rats are shown. d, e) Echocardiographic measurements of right ventricular end-diastolic diameter (RVEDD) and tricuspid annular plane systolic excursion (TAPSE) are shown for d) SuHx and e) Hx rats. Data are presented as mean±SD. *: p<0.05 *versus* baseline; **: p<0.01 *versus* baseline; ***: p<0.001 *versus* baseline.

Vessel morphology

In figure 6a, representative examples are shown of changes in 40-µm vessels in SuHx rats and more examples are presented in the online supplementary material. No changes in intima thickness were seen in arteries of 60–100 µm (fig. 6b), in contrast to an increase in intima thickness in smaller vessels (30–60 µm vessels in fig. 6c and <30-µm vessels in fig. 6d). Upon return to normoxia, the intima remained thickened in vessels with a diameter up to 60 µm, resulting in a progressive narrowing of the lumen. Media thickness

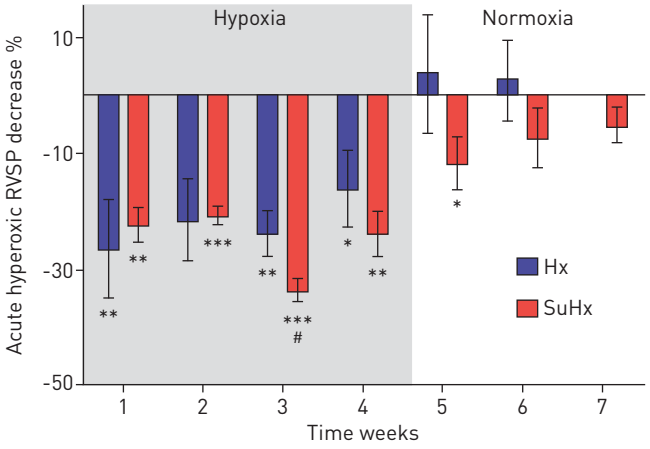

FIGURE 3 Right ventricular systolic pressure (RVSP) changes following an acute hyperoxic challenge in rats exposed to hypoxia only (Hx) and rats exposed to SU5416 and hypoxia (SuHx). Data are presented as mean±SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; #: $p<0.05$ *versus* Hx.

increased during the first week of hypoxia in <30- and 60–100-µm vessels, but decreased upon return to normoxia. A very strong correlation was observed between relative wall thickness (intima plus media) of <30-µm vessels and $\Delta RVSP_{max\text{-}min}$ (fig. 7).

Discussion

In the SuHx model, the combined exposure of rats to hypoxia and a VEGF-R antagonist induces angio-obliterative pulmonary hypertension [3]. By intima thickening mainly in the small arteries and limited neomuscularisation, the SuHx model reproduces many aspects of the pathobiology of human PAH [17]. Here, we evaluated the time-course of vascular remodelling by histology and the evolution of

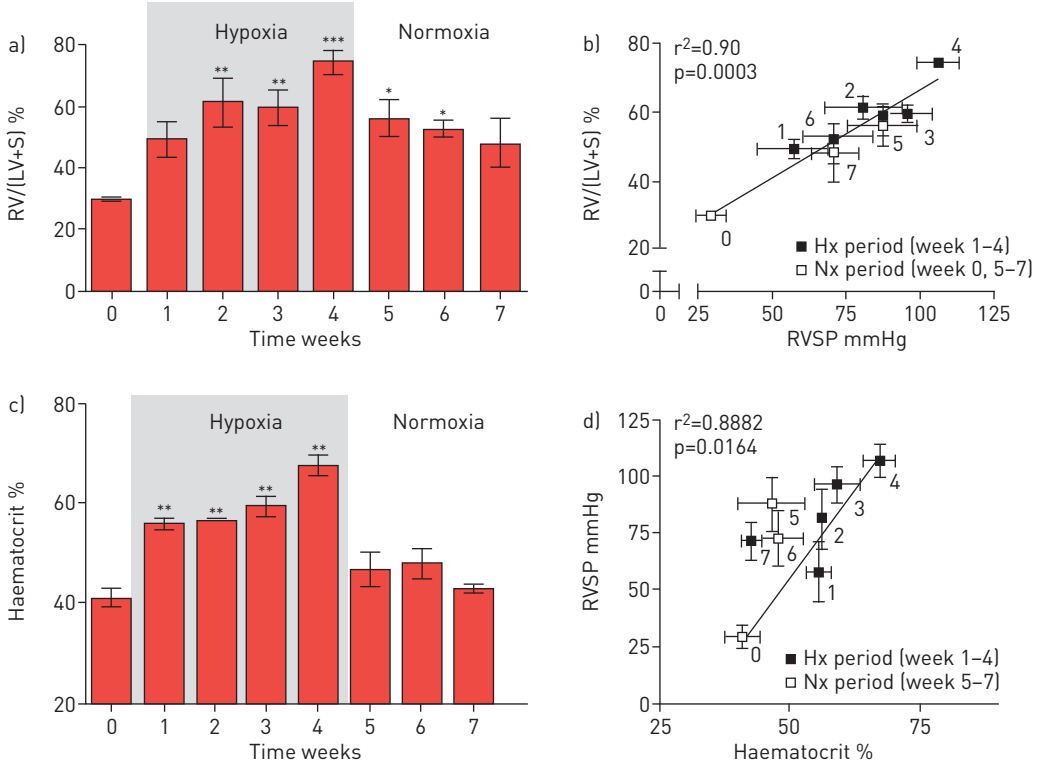

FIGURE 4 a) Weekly progression of right ventricular hypertrophy (expressed as right ventricular weight over left ventricular plus septal weight (RV/(LV+S))) in rats exposed to SU5416 and hypoxia (SuHx). b) There was a strong correlation between RV/(LV+S) in hypoxia (Hx) and after normoxic (Nx) re-exposure (determined in 32 rats from eight consecutive histology groups) and right ventricular systolic pressure (RVSP) (n=5; telemetry) at corresponding time-points (as indicated by study week number). c) Haematocrit of SuHx rats. d) Correlation, during the Hx period only, between haematocrit and RVSP (telemetry) at corresponding time-points (as indicated by study week number). RVSP and haematocrit were not related during the Nx re-exposure. Data are presented as mean±SD. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

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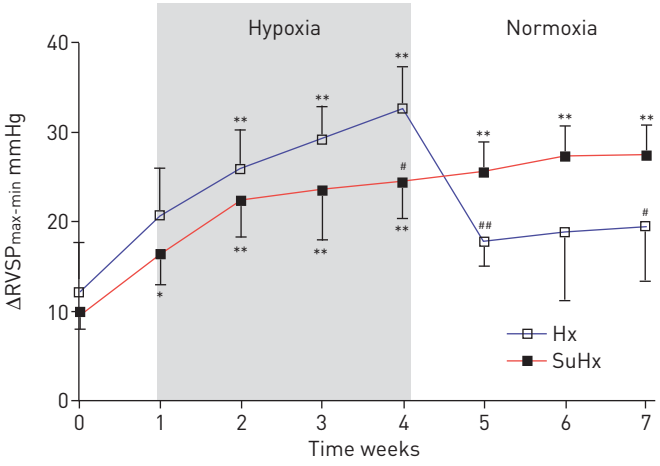

FIGURE 5 Daily ranges in right ventricular systolic pressure ($\Delta RVSP_{max\text{-}min}$) during the course of the experimental period in rats exposed to hypoxia only (Hx) and rats exposed to SU5416 and hypoxia (SuHx). $\Delta RVSP_{max\text{-}min}$ increased significantly during the hypoxic period in Hx and SuHx rats alike, but after return to normoxia, decreased in Hx rats and continued to increase in SuHx rats. Data are presented as mean±SD. *: $p<0.05$ *versus* baseline; **: $p<0.01$ *versus* baseline; #: $p<0.05$ *versus* Hx; ##: $p<0.01$ *versus* Hx.

haemodynamic changes using telemetry. Telemetry allowed several important observations in SuHx rats. First and surprisingly, the RVSP was higher than anticipated in conscious, freely moving Hx animals. Second, we showed that after the return to normoxia the SuHx model regresses to a milder but persistent form of pulmonary hypertension. Third, while acutely reversible hypoxic vasoconstriction is an important component of pulmonary hypertension during the first phase of the model, the second phase is characterised by intima remodelling, which was progressive even after the return to normoxic conditions. Fourth, we found a progressive increase in the $\Delta RVSP_{max\text{-}min}$ in SuHx rats, which can be used as a surrogate marker for the severity of vascular remodelling.

Serial haemodynamic and histological characterisation

Serial pressure measurements revealed an RVSP of 80 mmHg in Hx rats (and even higher in SuHx rats), which was higher than expected. Telemetry allowed measurements without anaesthesia, during physical activity and without the need to remove rats from their hypoxic environment. Although the *in vitro*

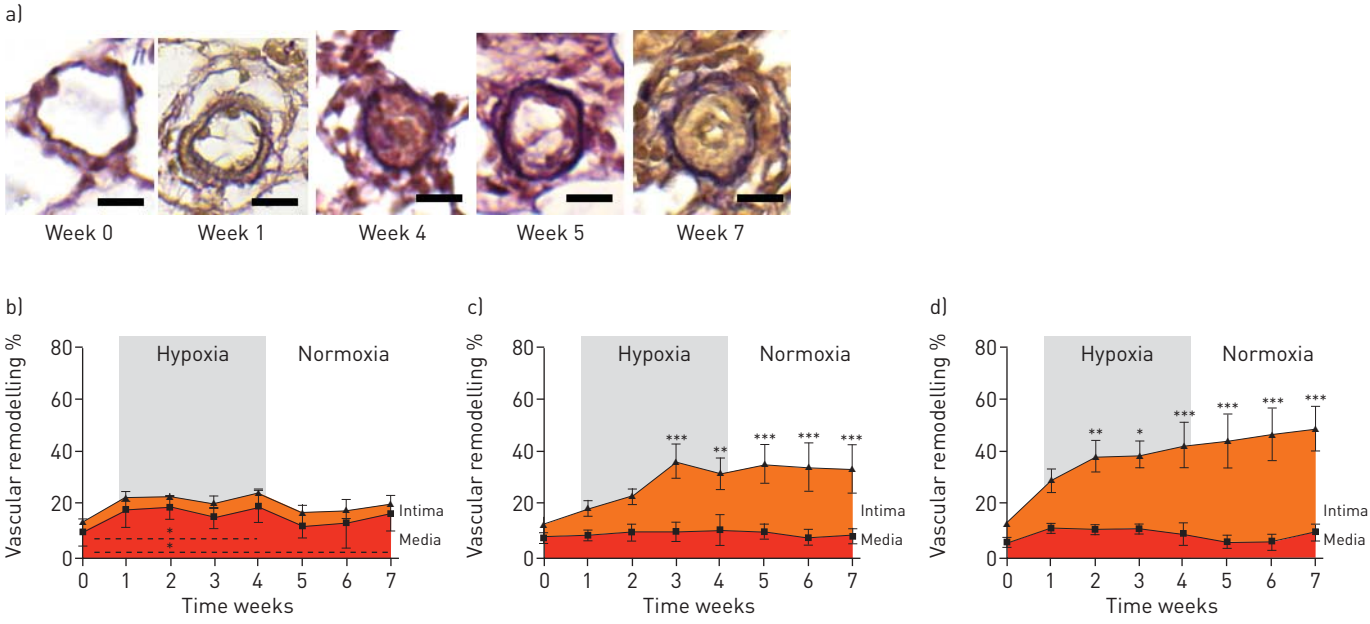

FIGURE 6 Histology of pulmonary vessels in rats exposed to SU5416 and hypoxia (SuHx). a) Representative Elastica–van Gieson stains of small pulmonary arteries (~40 µm) at different time-points (see the online supplementary material for more images). Time courses of the thickening of the intima and media were expressed as percentage of the diameter of the external elastic lamina and calculated per vessel class: b) 60–100-µm vessels; c) 30–60-µm vessels; d) <30-µm vessels. The medial layer was significantly thickened ($p<0.05$) during hypoxia and at week 7. Media and intima thickness are represented as mean±SD; completely obliterated vessels were also observed and included in the analyses. The media thickness of 60–100-µm vessels was significantly different between baseline and week 1 ($p<0.01$). Scale bars=20 µm. *: $p<0.05$ *versus* baseline by repeated ANOVA; **: $p<0.01$ *versus* baseline by repeated ANOVA; ***: $p<0.001$ *versus* baseline by repeated ANOVA.

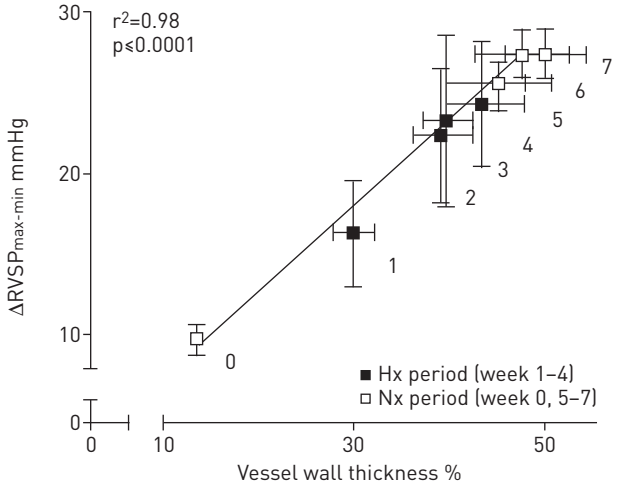

FIGURE 7 Pearson correlation between the relative vessel wall thickness (intima plus media) of the smallest vessel class (<30 μm) and the daily range in right ventricular systolic pressure ($\Delta RVSP_{max-min}$). Mean±SD values were taken from the histology and telemetry groups at corresponding time-points. The grey numbers indicate the week of data collection. Hx: hypoxia; Nx: normoxic.

induction of endothelial proliferation by SU5416 starts within 3 days [18], a trend toward significant haemodynamic differences between SuHx and Hx rats did not become apparent until after 2 weeks of hypoxic exposure. The development in SuHx rats of a RVSP that exceeded the pressure in Hx rats was paralleled by the emergence of intima remodelling and a decrease in vasoreactivity in week 4. Together, these findings suggest the emergence of vascular remodelling in SuHx rats 3 weeks after the administration of SU5416. As expected, RVSP in Hx rats decreased further, but not completely, to normal values in the successive weeks after the return to normoxia. The RVSP also decreased in SuHx rats upon return to normoxia, but stabilised at a new plateau (fig. 2). This up-and-partially-down pressure response in the SuHx model was not previously appreciated and contrasts with the findings of TOBA *et al.* [11], who showed persistent pulmonary hypertension in SuHx rats after return to normoxia. This difference is probably explained by the use of anaesthesia, because we also found much lower RVSPs in separate groups of anaesthetised SuHx rats prior to termination for tissue harvesting. Other than by the use of anaesthesia, differences in pressure responses between our study and the study by TOBA *et al.* [11] may be explained by differences in husbandry, animal handling and diet, including dietary copper content [19]. It is unlikely that technical complications of the telemetry method would explain differences in pressure responses, as the quality of all signals was carefully checked and signals in normal rats were stable. Clots at the site of telemetry implantation, which could possibly interfere with pressure recordings, were not observed. ABE *et al.* [8] followed SuHx-rats, without telemetry, up to 14 weeks after the initial SU5416 administration and showed that vascular remodelling was driven by vessel obliteration. This suggests that SuHx-induced pulmonary hypertension is not fully reversible and that a longer telemetric follow-up of our rats would have probably revealed stable or progressive pulmonary hypertension. The major implication of partial and temporal reversibility of pulmonary hypertension in SuHx rats is the fact that treatment studies need to be carefully planned and require sufficient numbers of animals to avoid any false positive results.

RVSP in SuHx rats with telemetry showed a linear correlation with RV/(LV+S) in SuHx rats without telemetry (fig. 4b). This confirms that telemetry implantation does not affect the development of experimental pulmonary hypertension [4, 12]. The haematocrit in SuHx rats increased significantly during the hypoxic period, which has been reported to be related to a combined effect of diuresis and intensified erythropoiesis [20]. The correlation between haematocrit and RVSP during the exposure to hypoxia suggests that some of the pressure increase in SuHx rats is related to an increase in blood viscosity. It is interesting that, despite the progressive increase in pulmonary vascular remodelling, right ventricular function (*e.g.* TAPSE) improves upon normoxic re-exposure. This suggests that hypoxic vasoconstriction and erythrocytosis significantly contribute to right ventricular remodelling in the initial stages of the model.

During the hypoxic exposure, Hx and SuHx rats exhibited a progressively greater circadian range in RVSP (fig. 5). Upon normoxic re-exposure, $\Delta RVSP_{max-min}$ recovered in Hx rats but not in SuHx rats. Interestingly, we demonstrated a strong correlation between $\Delta RVSP_{max-min}$ and wall thickness of <30-μm vessels, which suggests that $\Delta RVSP_{max-min}$ could be used as a marker of remodelling of resistance vessels. In this setting, the lung circulation fails to accommodate a temporary elevation in blood flow (which occurs during activity or stress), and as this class of vessels plays a major role in pulmonary vascular resistance [21, 22], RVSP increases substantially. This is in keeping with the previously published correlation between RVSP and the percentage of obliterative vessels [10, 14]. Further studies to understand the mechanism of this hemodynamic phenomenon and its response to treatment are warranted.

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Comparison to human PAH

Although the hyperproliferative characteristics of the pulmonary endothelium in the SuHx rat resemble similar changes in the human PAH lung, the mechanisms of action of SU5416 and hypoxia in this model have not been precisely identified. Therefore, potential treatment responses in this model may not be reproduced in human PAH. Unlike PAH patients, and despite severe pulmonary vascular remodelling and right ventricular dysfunction, SuHx rats exhibit low mortality rates. Serial histology of SuHx rats showed vascular remodelling predominantly of vessels up to 60 μm. Moreover, the tunica media was relatively unaffected. This reflects the findings in human PAH provided by STACHER *et al.* [17], who showed a large overlap between the media thickness of healthy controls and patients with end-stage human PAH. As such, end-stage human PAH and the SuHx model share features of fixed pulmonary hypertension and a histological pattern dominated by intima thickening. These changes may occur after an initial phase of vasoconstriction and media hypertrophy, and may explain why PAH-targeting treatments have varying degrees of success during distinct phases of the evolution of the pulmonary vascular disease in SuHx rats and PAH patients.

Use of telemetry

Telemetry has become the ''gold standard'' for blood pressure measurements in conscious, freely moving animals [23, 24], as continuous hemodynamic parameters can be acquired unaffected by the use of anaesthesia or mechanical ventilation. Here we show that telemetry is highly informative for the longitudinal tracking of the RVSP in SuHx-rats. Having established the natural history of the development of PAH in this model, it is now feasible to monitor the RVSP during treatment interventions. The magnitude of the circadian range in RVSP, as revealed by telemetry, underscores the importance of meticulous attention to measurement protocols when using animals for cardiovascular research. A disadvantage of the method is that reliable measurements could not be obtained past 8 weeks after the implantation of the transmitter, when ~70% of its battery life is consumed. When telemetric pressure measurement could be combined with cardiac output measurements, this would strengthen the benefit of this method for assessing treatment responses.

Conclusion

The SuHx rat model is a unique experimental model reproducing a significant number of pathobiologically important features of human PAH. The present study shows that in the model, an initial phase of partially reversible hypoxic vasoconstriction and polycythaemia is followed by severe pulmonary hypertension developing in association with progressive remodelling of the intima. Withdrawal from hypoxia is associated with rapid reversal of polycythaemia and a less rapid decrease in pressure, while pulmonary vascular remodelling progresses. Careful observation of the different phases of the SuHx model will now permit the testing of drugs during different stages of disease development. Telemetry studies, as described here in this animal model of severe PAH, can facilitate the design of preclinical studies to further improve our understanding of drug actions in PAH.

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