Haemodynamic unloading reverses occlusive vascular lesions in severe pulmonary hypertension

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Received 9 September 2015; revised 23 February 2016; accepted 17 March 2016; online publish-ahead-of-print 1 April 2016

Time for primary review: 41 days

Aims
An important pathogenic mechanism in the development of idiopathic pulmonary arterial hypertension is hypothesized to be a cancer-like cellular proliferation independent of haemodynamics. However, because the vascular lesions are inseparably coupled with haemodynamic stress, the fate of the lesions is unknown when haemodynamic stress is eliminated.

Methods and results
We applied left pulmonary artery banding to a rat model with advanced pulmonary hypertension to investigate the effects of decreased haemodynamic stress on occlusive vascular lesions. Rats were given an injection of the VEGF blocker Sugen5416 and exposed to 3 weeks of hypoxia plus an additional 7 weeks of normoxia (total 10 weeks) (SU/Hx/Nx rats). The banding surgery to reduce haemodynamic stress to the left lung was done at 1 week prior to (preventive) or 5 weeks after (reversal) the SU5416 injection. All SU/Hx/Nx-exposed rats developed severe pulmonary hypertension and right ventricular hypertrophy. Histological analyses showed that the non-banded right lungs developed occlusive lesions including plexiform lesions with marked perivascular cell accumulation. In contrast, banding the left pulmonary artery not only prevented the development of but also reversed the established occlusive lesions as well as perivascular inflammation in the left lungs.

Conclusion
Our results indicate that haemodynamic stress is prerequisite to the development and progression of occlusive neointimal lesions in this rat model of severe pulmonary hypertension. We conclude that perivascular inflammation and occlusive neointimal arteriopathy are driven by haemodynamic stress.

Keywords
Haemodynamic stress • Pulmonary arterial hypertension • Occlusive lesion formation

1. Introduction
Pulmonary arterial hypertension (PAH) is a diverse group of diseases characterized by progressive narrowing/occlusion of small pulmonary arteries (PAs), which results in increased pulmonary vascular resistance and severe pulmonary hypertension. Major factors contributing to the vascular narrowing are sustained vasoconstriction and structural remodelling.1 Despite recent advances in treatment, currently available therapies are unsatisfactory.2 Thus, a better understanding of the pathogenesis and more effective therapeutic options for PAH are imperative.

A current leading hypothesis of the pathogenesis of PAH, especially that of idiopathic PAH (IPAH), is that after having multiple insults, cells in the small PAs undergo phenotypic changes independent of haemodynamics to become apoptosis-resistant and highly proliferative, which result in severe luminal narrowing/occlusion (Figure 1).3–5 This concept is indirectly supported by clinical findings, such as ineffectiveness of vasodilators acutely as well as chronically,2 and the appearance of highly
proliferative apoptosis-resistant cells in the remodelled PAs.\textsuperscript{5–8} However, there are still uncertainties regarding this concept, because we have never been able to test experimentally what would happen to the established occlusive neointimal lesions if haemodynamic stress were eliminated. Indeed, single lung transplantation\textsuperscript{9,10} or main pulmonary artery banding\textsuperscript{11} was reported to regress occlusive lesions in PAH, although the degrees of regression varied depending likely on the degree and duration of the flow reduction to the hypertensive arteries.

Recent studies highlight chronic inflammation as a key factor in the pathogenesis of PAH. In addition, it has been reported that abnormal haemodynamic stress can induce inflammatory responses in the pulmonary as well as the systemic circulation.\textsuperscript{12–14} We, therefore, hypothesize that in PAH severe sustained haemodynamic stress is essential for the development and progression of occlusive pulmonary arterial remodelling via induction of perivascular inflammation (Figure 1).

To test this hypothesis, we used a combination of two established models, left pulmonary artery banding (LPAB) to decrease left pulmonary blood flow and haemodynamic stress (haemodynamic unloading)\textsuperscript{15,16} and the Sugen5416/hypoxia/normoxia (SU/Hx/Nx)-exposed rat, which closely mimics human IPAH histologically and haemodynamically.\textsuperscript{17,18}

2. Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Kyushu University, Japan, and all animal procedures were performed by following the principles of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011).

2.1 SU5416/hypoxia/normoxia (SU/Hx/Nx)-exposed rats

Adult male Sprague–Dawley rats weighing 200–250 g were given a subcutaneous injection of SU5416 (20 mg/kg, Cayman Chemical, MI, USA) and exposed to hypoxia (10% O\textsubscript{2}) for 3 weeks and then returned to normoxia (21% O\textsubscript{2}) for an additional 2–7 weeks.\textsuperscript{17}

2.2 Left pulmonary artery banding

The LPAB surgery in rats was performed as described previously with minor modifications.\textsuperscript{15,16} Briefly, under anaesthesia with pentobarbital sodium (30 mg/kg, IP), rats were intubated and ventilated during the whole procedure (tidal volume, 1.0 mL; respiratory rate, 100/min). Inhalation anaesthesia with isoflurane was used to maintain a surgical level of anaesthesia. Under sterile conditions, a left thoracotomy was performed in the third intercostal space followed by the section of the intercostal muscles. The pleura were opened and the left lung mobilized. The left hilum was located, and a 4-0 silk thread was positioned under the left pulmonary artery. The suture was tied tightly around a 25-gauge needle (outer diameter, 0.51 mm) that was placed alongside the left pulmonary artery. Then, the needle was rapidly removed in order to produce a fixed constricted opening in the lumen equal to the diameter of the needle. Subsequently, the chest was closed with 2-0 polydioxanone loop for intercostal spaces. Muscle and skin layers were closed with 4-0 polypropylene. Rats were disconnected from ventilator. Upon confirmation of spontaneous respiration, the animal was extubated.

2.3 Experimental protocols and groups

We had five experimental rat groups; normal and SU/Hx/Nx-exposed rats with and without LPAB at various time points to look at the effects of haemodynamic unloading on lung histology and inflammatory signalling. In the preventive protocol, LPAB was performed 1 week prior to the injection of SU5416 to test if haemodynamic stress contributes to the development of occlusive lesions. In the reversal protocol, we performed LPAB at 5 weeks after the initiation of PAH, because our detailed temporal analyses of this model indicate that RVSP peaks with a decline in cardiac output and numerous small PA occlusive lesions are formed at this time point.\textsuperscript{18} Haemodynamic and histological analyses were done at 10 weeks after the SU5416 injection for both protocols, because this timing is an advanced stage of the SU/Hx/Nx-exposed rats when the PAH rats without LPAB develop a sustained very high RVSP and extensive occlusive neointimal lesions including concentric occlusive and plexiform lesions.\textsuperscript{18} In a separate series of experiments, we examined effects of a short-term LPAB on established PA occlusive lesions and inflammatory responses. We performed LPAB at 5 weeks after the initiation of PAH, and haemodynamic measurements were then performed at 3 days, instead of at 5 weeks as in the reversal protocol, after the LPAB surgery (3-day reversal). We had five groups of rats: (i) age-matched normal controls; (ii) 5-week SU/Hx/Nx-exposed rats; (iii) 10-week SU/Hx/Nx-exposed rats with LPAB surgery (preventive protocol) and (iv) 10-week SU/Hx/Nx-exposed rats with LPAB surgery at 5 weeks (reversal protocol); and (v) 3-day reversal (Figure 2).

At the end of each haemodynamic study, all rats except for the 3-day reversal group for which haemodynamic measurements were not performed were euthanized by an overdose of pentobarbital sodium, and the hearts were excised. The weight ratio of right ventricle/left ventricle (RV/LV) \(\text{+ septum} = \text{RV/LV + S} \) was obtained for each animal as an indication of RV hypertrophy. The lungs were inflated with 10% formalin plus 0.5% agarose at 20 cm H\textsubscript{2}O pressure, and fixed in 10% formalin overnight.\textsuperscript{17} The left and right lobes were blocked and paraffin embedded. All sections were cut at 5 \(\mu\)m and were stained with haematoxylin and eosin (H&E) or Verhoeff–Van Gieson.

2.4 Measurements of pulmonary blood flow

The ratio of pulmonary blood flow to the left and the right lungs was estimated using microspheres (Dye-Trak VII, Triton Technology Inc.).\textsuperscript{19} The lungs were isolated from the anaesthetized rats at one day after LPAB and its sham-operation. The lungs were ventilated with room air, and perfused at constant-flow rate with physiological salt solution as described previously.\textsuperscript{20} The microspheres (10 000 particles per 100 mL) were added to the perfusate reservoir and circulated through the lungs. The lungs were digested and the microspheres trapped in the left and right lungs were counted by spectrometer as described previously.\textsuperscript{19}

2.5 Haemodynamic measurements in catheterized rats

Rats were placed on a controlled heating pad after they were anaesthetized with pentobarbital sodium (30 mg/kg, IP). Haemodynamic measurements...
were performed under normoxic conditions. Briefly, a polyvinyl catheter (PV-1, internal diameter: 0.28 mm) was inserted into the right ventricle (RV) via the right jugular vein for measurement of right-ventricular systolic pressure (RVSP).21 RVSP was generally measured to estimate systolic pulmonary arterial pressure, because catheterization of the pulmonary artery was often difficult in these extremely hypertensive (RVSP > 80 mmHg) rats.20 A microtip P-V catheter (FTH-1912B-8018, Transonic Inc.) was inserted into the right carotid artery to measure mean systemic arterial pressure. The signals were continuously recorded by ML880/9 PowerLab 16/30 (AD Instruments) with Science ASVantage 5.0 control unit (FY097B, Transonic Inc.) and a personal computer. Heart rate was monitored to be consistent (>300 bpm).20

2.6 Histopathological and immunohistochemical analysis

2.6.1 Luminal occlusive lesions
All small PAs (more than 100 vessels per cross section of left and right lobes including the hilum, outer diameter < 100 μm) were evaluated by at least three investigators who were unaware of the source of the sections.18,21 Vessels were assessed for occlusive neointimal lesions on Verhoeff–van Gieson stained slides and scored as: no evidence of neointimal formation (Grade 0), partial luminal occlusion (< 50%, Grade 1), and severe-luminal occlusion (≥ 50%, Grade 2). PA occlusion rate was expressed as percentage of each grade. We assessed the vessels of outer diameter < 50 μm and between 50 and 100 μm as described previously.21

2.6.2 Perivascular cell counts
Perivascular cell infiltration was defined as the number of nuclei within an imaginary 100 μm-diameter circle around the vessel in H&E-stained lung tissue sections from five experimental groups.22 All cells were counted in 15 random fields in an uninformed manner.

2.6.3 NFκB activity
A redox-sensitive transcription factor, nuclear factor κB (NFκB), is known as one of the key inflammatory markers in the development of PAH.23 Immunohistochemical staining of NFκB was performed using the Vectastain Universal Quick kit (Vector Laboratories).17 The αp65 mAb of NFκB (Cell Signaling) which recognizes an epitope on the p65 subunit that is masked by bound inhibitor of κB (IκB), detects activated NFκB. An uninformed observer counted the number of NFκB-positive cells in 10 random fields at a magnification of ×400.

2.6.4 Macrophage accumulation
Macrophages were detected by immunostaining by anti CD68 antibody (Abcam). An uninformed observer counted the number of CD68-positive cells in 10 random fields at a magnification of ×400.

2.6.5 Proliferation
The degree of proliferative cells was evaluated by anti Ki67 antibody (Thermo Scientific). An uninformed observer counted the number of Ki67-positive cells in 10 random fields at a magnification of ×400.

Figure 2. Experimental protocols and groups. LPAB, left pulmonary artery banding; P, rats with LPAB in the prevention protocol; R, rats with LPAB in the reversal protocol; 3R, rats with LPAB in the 3-day reversal protocol.
2.7 Reverse transcription–PCR analysis of cytokine expression in lungs

In order to evaluate the effects of LPAB on pro-inflammatory cytokines, we measured the gene expression of IL-1β, IL-6, and TNFα in lungs of the following groups: (i) age-matched normal controls (left lungs), (ii) 5-week SU/Hx/Nx-exposed rats (left lungs), and (iii) 10-week SU/Hx/Nx-exposed rats with LPAB surgery at 5 weeks (reversal protocol) (left and right lungs separately). Total RNA was extracted by using RNeasy fibrous Tissue Mini Kit (Qiagen). The primers used in reverse transcription–PCR (RT–PCR) analysis of rat IL-1β, IL-6, and TNFα were purchased from Genenet Co. (Table 1). One microgram total RNA was subjected to the 10 μL RT reaction using ReverTra Ace qPCR RT Kit (Toyobo). Aliquot of RT product was diluted 10 times with water and 2 μL of the dilution was subjected to a real-time PCR analysis using a SYBR Premix Ex Taq™ II (TAKARA) and a LightCycler (Roche, Basel, Switzerland). The thermal cycle profile used for the amplification comprised the initial denaturation at 94°C for 10 min and the following 40 cycle amplification step of denaturation at 94°C for 10 s, annealing at 60°C for 10 s and extension at 72°C for 30 s. The melting curves of the PCR product were analysed at the end of the real-time PCR analysis. The melting curve analysis confirmed the single peak of the melting profile. Electrophoresis confirmed that each PCR product showed a single band. The acquired fluorescence data were analysed with ΔΔCt method, with 18S as an internal control.

2.8 Statistical analysis

Values are means ± SEM. Comparisons between groups were made with Student’s t-test or ANOVA with Scheffe’s post hoc test for multiple comparisons. Differences were considered significant at P < 0.05.
consistent with our previous findings. In the prevention protocol, the non-banded right lungs developed more severe occlusive lesions including mature plexiform lesions (Figure 4A, F, and G) at 10 weeks than those at 5 weeks after SU5416 injection, whereas the banded left lungs showed a striking contrast, i.e. they appeared histologically nearly normal, including vascular and airway structures (Figure 4B, F, and G).

In the reversal protocol, the non-banded right lungs developed severe occlusive lesions similar to those in the prevention protocol.
(Figure 4C, F, and G), while such lesions nearly completely disappeared in the banded left lungs (Figure 4D, F, and G).

3.4 Effects of LPAB on perivascular inflammatory cell accumulation and cell proliferation

We found many mononuclear cells accumulated around the occluded PAs in lungs from rats at 5 weeks after SU5416 injection without LPAB (Figure 5B and A). The perivascular cells were predominantly macrophages (Figure 5H and B). The accumulated numbers of perivascular cells including macrophages were increased in the non-banded right lungs in both the prevention and the reversal protocols (Figure 5C, I, E, K, A, and B). These accumulations of perivascular cells were decreased to normal levels in the banded left lungs (Figure 5D, J, I, A, L, and B). Immunostaining with nuclear factor κB-αp65 (NFκB activity) showed that the majority of these perivascular cells and some intimal cells were highly positive at 5 weeks after SU5416 injection without LPAB (Figure 5N and C) and in the non-banded right lungs in both the prevention and the reversal protocols (Figure 5O, Q, and C), whereas the staining of these cells was decreased to normal levels in the banded left lungs (Figure 5P, R, and C). The staining pattern of Ki67 positive cells was somewhat similar, although less frequent, to that of NFκB-αp65, i.e. many perivascular and some limited intimal cells are stained positive with Ki67 at 5 weeks after SU5416 injection without LPAB (Figure 5T and D) and in the non-banded right lungs in both the prevention and the reversal protocols (Figure 5U, W, and D). The Ki67 positive cell numbers were near normal levels in the banded left lungs in both experimental protocols (Figure 5V, X, and D).

3.5 Effects of LPAB on cytokine mRNA expression

As shown in Figure 6A, mRNA levels of IL-6 were significantly elevated in lungs at 5 weeks after SU5416 injection without LPAB compared with normal controls. In the reversal protocol rats, the expression of IL-6 remained high in the non-banded right lungs but was significantly reduced by LPAB. In contrast, although mRNA levels of IL-1β and TNFα were significantly higher in lungs at 5 weeks after SU5416 injection without LPAB than in normal controls, the high expression levels appeared to be spontaneously reduced in the non-banded right lungs at 10 weeks in the reversal protocol rats, and no significant effects of LPAB were observed in the expression levels of IL-1β and TNFα (Figure 6B and C).

3.6 Effects of a short-term (3 days) LPAB

As shown in Figure 7, NFκB-αp65 expression decreased markedly, and the number of accumulated perivascular cells had also already decreased significantly in banded left when compared with non-banded right lungs after a relatively short period (3 days) of the LPAB. In contrast, the occlusion rate in small PAs did not differ between 3-day banded and non-banded lungs.

4. Discussion

The role of haemodynamic stress in the pathogenesis of occlusive neointimal lesions in PAH, especially in IPAH, is controversial, although its significance in the increased flow-associated PAH in patients with congenital heart disease has been well documented. Here, we directly addressed this important issue, i.e. what happens to the occlusive neointimal lesions in small PAs when haemodynamic stress is reduced in an experimental model of IPAH. The most striking finding of this study is that LPAB (haemodynamic unloading) nearly fully reversed established occlusive PA lesions and perivascular inflammation in advanced pulmonary hypertension. This result supports the case reports after single lung transplantation in PAH patients, and provides substantial experimental evidence that haemodynamic stress is essential for the maintenance/progression of severe occlusive small PA lesions and perivascular inflammatory cell accumulation.

Our results of the prevention study support the hypothesis that haemodynamic unloading prevents development of severe occlusive neointimal lesions and inflammation in SU/Hx/Nx-exposed PAH rats. Because LPAB allows us to separate the effects of haemodynamic stress and the other factors in the same animal, in this case VEGF receptor blockade (SU) and hypoxia (Hx), differences observed in the left and the right lungs can be attributed to the differences in haemodynamic stress. Therefore, a major mechanistic implication is that SU/Hx-induced haemodynamic stress (vasoconstriction and hypertension) rather than the combination of specific stimuli, SU/Hx per se, is the factor necessary for the occlusive PA remodelling, which is in agreement with the findings of PA medial remodelling (muscularization) in chronically hypoxic and monocrotaline-exposed rats. In other words, any combination of stimuli that results in sustained severe haemodynamic stress (presumably in the case of SU/Hx, the combination of hypoxia-induced pulmonary vasoconstriction and SU-induced endothelial apoptosis and dysfunction that exaggerates the vasoconstriction) can induce the PAH neointimal vascular phenotype. This may explain at least partly why nearly all congenital heart disease cases under certain conditions, i.e. non-restricted and post-tricuspid shunt (high flow and large pressure gradient), develop PAH and the plexogenic arteriopathy.

The findings that a short-term (3 days) restriction of haemodynamic stress significantly reduced the inflammatory responses (perivascular cell accumulation and NFκB activation) with no obvious change in the occlusive remodelling suggest that inflammation is a downstream phenomenon of haemodynamic stress and likely upstream of vascular remodelling. We next addressed a question of which inflammatory mediators are involved in the vascular remodelling. We observed that lung mRNA levels of IL-6, IL-1β, and TNFα were increased at the 5-week time point of the SU/Hx/Nx-exposed rats. The high levels of IL-6 were sustained at later time points, while those of IL-1β and TNFα declined over time. This is consistent with the findings of a previous report of inflammatory signalling in SU/Hx/Nx rats. We also found that the sustained high expression of IL-6 as well as activity of NFκB were markedly attenuated by LPAB, suggesting that the haemodynamic stress sensitive NFκB-IL-6 pathway activity may be a central player in the maintenance of inflammation in the severe occlusive arteriopathy in this model. Although further studies are needed to identify the detailed regulatory relationships between NFκB and pro-inflammatory cytokine activities, the concept that the NFκB-IL-6 signalling pathway is important in mediating pulmonary vascular inflammation agrees with that in the systemic circulation. A remaining and perhaps most important question is what mechanism haemodynamic stress directly activates to induce the downstream perivascular inflammatory process.

We observed that many SU/Hx/Nx-exposed rats with LPAB developed overt right heart failure, unlike those without LPAB. We speculate that the non-banded right lungs of SU/Hx/Nx-exposed rats with
LPAB developed more rapid and severe pulmonary hypertension due to an additional factor/hit (increased haemodynamic stress) to the original two—VEGF receptor blocker and hypoxia. This speculation is supported by the observation that the mortality rate of SU/Hx/Nx-exposed rats without LPAB increases after the PAH rats are exposed to treadmill exercise (increase in cardiac output).\textsuperscript{31}
A very intriguing question this study raises is whether or not the occlusive PA lesions return upon re-exposure to normal haemodynamic stress. In other words, are histologically near-normal left-banded lungs functionally normal? We tried to answer this question by reopening the banded LPA, but found removing the suture 5 weeks after the banding was technically unrealistic because of the tissue adhesions and

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**Figure 6** Effects of left pulmonary artery banding on pro-inflammatory cytokine expression. The gene expression levels of pro-inflammatory cytokines in lungs from normal and SU/Hx/Nx-exposed rats at the 5-week time point without LPAB and left and right lungs from the reversal protocol rats. Nor: normal, R: rats in the reversal protocol. Values are means ± SE of n = 7–8 each. *P < 0.001 vs. Nor. †P < 0.05 vs. 5W. ‡P < 0.001: right vs. left lungs in reversal protocol.

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**Figure 7** A short-term left pulmonary artery banding attenuates perivascular inflammation but not occlusive vascular remodelling. Representative low magnification photomicrographs showing NFκB-αp65-stained right (non-banded, A) and left (banded, B) lungs at 3 days after left pulmonary artery banding surgery in established PAH (5 weeks after Sugen5461 injection) rats (see insets for higher magnification images). (C) Percentages of grade 0 (white), 1 (grey), and 2 (black) pulmonary arterial occlusions of outer diameter (OD) of ≤50 μm (left) and 50 < OD < 100 μm (right). Statistical analysis of the counting results for total perivascular (D) and NFκB-αp65-positive cell number (E). 3R: rats with left pulmonary artery banding in the 3-day reversal protocol. HPF: high-power field (a magnification of ×400). Scale bars indicate 200 μm. Values are means ± SE of n = 4. *P < 0.05: Right vs. Left.
increased connective tissues around the suture. Considering that the initial combination of insults (single injection of SU and exposure to Hx) no longer exists after rats are re-exposed to normoxia (this may also be the case for most IPAH cases but possibly not for heritable PAH patients who carry genetic abnormalities), there is a possibility that sustained high haemodynamic stress is the sole driving force to maintain inflammation and severe vascular remodelling, creating a vicious cycle (Figure 1), which is similar to what happens in congenital heart disease associated PAH. Although this issue needs to be further investigated, if the lesions did not return upon re-exposure to normal haemodynamic stress, then the potential translational significance of this study would be enormous. We may be able to repair PAH lungs and generate near normal lungs and a possible cure of the disease (it would be virtually like performing a single lung transplantation) by haemodynamic unloading (resting the damaged/remodelled pulmonary circulation). Although the unilateral pulmonary artery banding in advanced IPAH patients is clinically unrealistic because of the likely development of right heart failure, there are potential ways to practically achieve the haemodynamic unloading/resting of the pulmonary circulation. For example, it can be done by using extracorporeal membrane oxygenation (ECMO). Recent reports indicate that the Novalung® device (a pulsatile pulmonary artery to left atrium ECMO) can be used safely for several weeks (up to 174 days) in PAH patients awaiting lung transplantation.32

We performed the LPAB at the 5-week time point in the reversal protocol for the following reasons. First, as we reported previously,16 based on the comparison of haemodynamic progression profile of SU/Hx/Nx-exposed rats with human PAH, around the 5-week time point when RVSP peaks and cardiac output begins to decline in the rat model may correspond roughly to the time of diagnosis in humans. Although mature complex plexiform lesions are rarely observed in the rat model at this time point,17,18 this may be the case with human PAH at the time of diagnosis.33 Secondly, when we attempted to determine whether mature plexiform lesions are reversible with haemodynamic unloading by performing the LPAB at a later time point (8-week), we found that these rats did not survive the open chest surgery and LPAB (a sudden increase in RV afterload) because the RV dysfunction is too advanced at this point in time. Taken together, our findings support that perivascular inflammation and occlusive arteriopathy in PAH are driven by haemodynamic stress (Figure 1). Once haemodynamic stress induces occlusive lesions, presumably via the activation of inflammation, these lesions further increase haemodynamic stress leading to worsening of the occlusive lesions. Therefore, the haemodynamic stress and occlusive lesions form a distinct vicious cycle. This may explain partly why PAH autonomously worsens with time. This also suggests the possibility of a novel therapeutic strategy that the vicious cycle could be reversed to a virtuous cycle, if we can intervene in the haemodynamic stress as discussed earlier, or possibly by eliminating the inflammatory process.

In conclusion, this study provides solid experimental evidence that haemodynamic stress is prerequisite to the development and progression of the perivascular inflammation and occlusive arteriopathy of PAH. This information will impact the field of pulmonary hypertension research and treatment by redirecting the attention of investigators to what is now regarded by many as a less important pathogenic factor, i.e., haemodynamic stress.

Acknowledgements
We thank Akiko Ando and Kazuhiro Kamada for their technical assistance and Kaori Oshima for helpful comments.

Conflict of interest: none declared.

Funding
This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion Science (20588107, 24659393, 24390198, 23220013) and, in part, a grant from Actelion Pharmaceuticals Japan Inc.

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