Macitentan reverses early obstructive pulmonary vasculopathy in rats: early intervention in overcoming the survivin-mediated resistance to apoptosis

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Shinohara T, Sawada H, Otsuki S, Yodoya N, Kato T, Ohashi H, Zhang E, Saitoh S, Shimpo H, Maruyama K, Komada Y, Mitani Y. Macitentan reverses early obstructive pulmonary vasculopathy in rats: early intervention in overcoming the survivin-mediated resistance to apoptosis. Am J Physiol Lung Cell Mol Physiol 308: L523–L538, 2015. First published December 24, 2014; doi:10.1152/ajplung.00129.2014.—It remains unknown whether current disease-targeting therapy can histologically reverse obstructive pulmonary vasculopathy and how the timing of the therapy influences the antiremodeling effects of the compound. We test the hypothesis that a novel endothelin receptor antagonist macitentan reverses the early and/or late stages of occlusive pulmonary vascular disease (PVD) in rats. Rats with pulmonary arterial hypertension (PAH), which were produced by combined exposure to a vascular endothelial growth factor receptor inhibitor Sugen 5416 and hypoxic hypoxia for 3 wk, were assigned to receive macitentan or vehicle during 3–5 wk (early study) or during 5–8 wk (late study) after Sugen injection. Compared with vehicle-treated PAH rats and PAH rats evaluated before treatment initiation, the macitentan-treated rats showed decreases in the proportion of occlusive lesions in the early study, a finding consistent with the reversal of right ventricular systolic pressure and indexes of right ventricular hypertrophy and medial wall thickness. Macitentan ameliorated but did not reverse the proportion of occlusive lesions in the late study. Although macitentan decreased the proportion of Ki67+ lesions in both studies, macitentan increased the proportion of cleaved caspase 3+ lesions and suppressed an antiapoptotic molecule survivin expression in the early study but not in the late study. In conclusion, macitentan reversed early but not late obstructive PVD in rats. This reversal was associated with the suppression of survivin-related resistance to apoptosis and proliferation of cells in PVD.

PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive obstructive pulmonary vasculopathy that leads to increased pulmonary vascular resistance, right ventricular failure, and premature death. This disorder is characterized by an imbalance in proliferation/apoptosis, a resistance to apoptosis in vascular cells, and later appearance of fibrotic change in the lesions (6). Recently, a significant body of evidence, including the results of meta-analyses, showed that drugs targeting one or more of three principal pathways, the endothelin, nitric oxide, and prostacyclin pathways, can improve clinical and hemodynamic parameters and even the survival of patients with PAH (10). However, it remains unknown whether any current PAH-specific compounds can histologically reverse obstructive pulmonary vasculopathy; the underlying mechanisms that may be involved remain unclear. Furthermore, disease progression occurs despite the availability of drugs specific for the disorder, which may, in part, be related to the late initiation of the therapy (2, 11, 30, 35). However, how the timing of the therapy influences the antiremodeling effects of the compound and the associated mechanisms remains unknown.

Recently, a new human PAH-like rat model, in which a single injection of a vascular endothelial growth factor (VEGF) receptor blocker Sugen 5416 in combination with chronic hypoxia produced human-PAH-like lesions, cellular intimal hyperplasia, intimal fibrosis, and plexiform lesions, was reported (1, 13). Pulmonary vasculopathy in this Sugen/hypoxia (SuHx) model is characterized by the progressive obstructive disease and the later appearance of intimal fibrosis, which was not produced in conventional PAH models. This pulmonary vasculopathy was further characterized by an imbalance in proliferation/apoptosis in the occlusive lesions (4, 19, 25, 37). However, it remains unknown whether the already established histological changes in this model can be reversed by any drugs at any stage of disease. Furthermore, although the pivotal role of apoptosis related to survivin, an antiapoptotic molecule, and caspase 3, a downstream molecule, has been demonstrated in the reverse remodeling process in monocrotaline-treated PAH rats and in a vascular injury model (3, 22), it is unknown whether suppression of survivin-mediated resistance to apoptosis is associated with anti remodeling effects of any compound in SuHx rats in vivo (4). Very recently, a dual endothelin receptor antagonist, macitentan, was developed (15). Preclinical studies suggested that macitentan has improved efficacy relative to that of other endothelin receptor antagonists bosentan and ambrisentan, which may be related to the better tissue penetration and binding kinetics of this compound (15, 39). The phase 3 trial, SERAPHIN, reported that long-term treatment with macitentan significantly decreased morbidity and mortality in patients with PAH, in addition to improving other clinical and hemodynamic parameters (27). However, the impact of this efficacious compound on occlusive pulmonary vasculopathy in SuHx PAH rats and the associated mechanisms are unknown.

We therefore tested the hypothesis that macitentan reverses occlusive pulmonary vascular disease at an early and/or late stage of disease in this model. We next investigated whether the imbalance of proliferation/apoptosis and resistance to apoptosis were appropriately regulated by macitentan, relative to any reverse remodeling effects of the compound in vivo.

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METHODS

Study design. The protocols for all animal experiments were approved by the Animal Research Committee, Mie University School of Medicine. Seven-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 210–250 g were used for the experiments. The animals were housed in standard laboratory conditions and fed a laboratory diet and water ad libitum. All rats were weighed weekly. To establish experimental PAH, the rats were subcutaneously injected with a VEGF receptor tyrosine kinase inhibitor Sugen 5416 (20 mg/kg; Tocris Bioscience, Bristol, UK) and exposed to hypobaric hypoxia.

Fig. 1. Study design. A outlines the early study (n = 40); B, the late study (n = 38). Black arrowhead indicates date for the assessment of outcome parameters. White arrowhead indicates the date of the harvest for the mechanistic study (n = 20). SuHx, combined exposure to Sugen 5416 and hypobaric hypoxia for 3 wk in rats; w, weeks.
hypoxia (10% O₂) for 3 wk (1, 26, 36). The rats were subsequently returned to ambient air (21% O₂) and maintained until designated time points. To investigate whether macitentan reverses the early and/or late stage of obstructive pulmonary vasculopathy, macitentan was administered during weeks 3–5 (early intervention study) and weeks 5–8 (late intervention study) after Sugen 5416 injection. A baseline SuHx PAH rat group, euthanized just before treatment initiation, was present in the early and late studies to determine the reversal of disease during treatment. Macitentan (Actelion Pharmaceuticals, Allschwil, Switzerland) was suspended in methylcellulose [0.5% (wt/wt) methylcellulose, 0.05% (vol/vol) Tween 80 in deionized water] (15), and 30 mg/kg of the compound was administered once daily by oral gavage on 7 days a week.

Treatment protocol for each study group was schematically outlined in Fig. 1. Specifically, in the early intervention study (n = 40) (Fig. 1A), SuHx rats were administered either macitentan (30 mg/kg) (SuHx 3 w + Macitentan 2 w) or vehicle (SuHx 3 w + Vehicle 2 w) for 2 wk and were compared with baseline PAH rats euthanized just before treatment initiation (SuHx 3 w) and normal control rats that were injected with diluent, kept in the ambient air, and treated with vehicle for 2 wk. In the late intervention study (n = 38) (Fig. 1B), after being kept in the ambient air for an additional 2 wk, SuHx rats were treated with either macitentan (SuHx 5 w + Macitentan 3 w) or vehicle (SuHx 5 w + Vehicle 3 w) for 3 wk and were compared with baseline PAH rats (SuHx 5 w) and control rats. Outcome parameters were assessed 24 h after the last treatment in the early and late studies.

To investigate in vivo mechanisms involved in reverse remodeling effects of the compound, SuHx PAH rat groups were treated similarly with macitentan or vehicle for 3 days and euthanized 6 h after the third treatment in the additional studies for the early (SuHx 3 w + Macitentan or Vehicle 3 d) and late (SuHx 5 w + Macitentan or Vehicle 3 d) interventions.

**Hemodynamic measurements.** Twenty-four hours after the last treatment, transthoracic closed-chest echocardiography was performed by Nemio 35 ultrasound device (Toshiba, Tokyo, Japan) with a 14.0-MHz linear transducer under pentobarbital sodium (33 mg/kg, intraperitoneal injection) anesthesia. A depository cream was used to remove hair from the chest area and ultrasound transmission gel was applied to the transducer and spread over the chest. Transthoracic two-dimensional and pulsed-wave Doppler modes were used to obtain the triplet of main pulmonary artery (PA) diameter measurements and the five velocity-time integral measurements close to the center of the vessel. Echocardiographically derived cardiac output was calculated with the formula [(main PA area) × (main PA velocity-time integral) × heart rate] as published previously (29).

The same rats were subsequently catheterized by the closed-chest technique. Briefly, a catheter of silicone elastomer tubing (inside diameter, 0.31 mm; outside diameter, 0.64 mm) was inserted into the right ventricle (RV) or the aorta through the right external jugular vein or the right carotid artery, respectively, as described previously (23, 24, 32). RV or aortic pressure was monitored with a physiological transducer (Uniflow, Baxter International, Deerfield, IL), an amplifier system (AP-620G, Nihon Kohden, Tokyo, Japan), and a monitor (Polygraph system, Nihon Kohden).

**Tissue preparation.** After hemodynamic measurements, the rats were mechanically ventilated and a midline sternotomy was performed to expose the heart and lungs (23, 24, 32). The right peripheral lung sections were excised for gene and protein expression analysis. The left lungs were quickly perfused through a PA cannula with phosphate-buffered saline. Then the isolated lungs were distended until fully inflated and fixed by perfusion through a tracheal tube and the PA cannula with 4% phosphate-buffered paraformaldehyde. The lungs were then clamped and maintained in fixative at 4°C for 3 h. Phosphate-buffered paraformaldehyde-fixed paraffin sections ob-
tained from the midsection of the left lung were used for pathological analysis. The RV was dissected from the left ventricle plus septum (LV+S) and weighed separately. The weight ratio [RV/(LV+S)] was calculated.

Assessment of occlusive lesions. Elastic Van Gieson (EVG) staining was performed on 5-µm sections. The microscope slides were analyzed in a blinded fashion without knowledge of the treatment groups. All small arteries [outer diameter (OD), 15–50 µm] per lung section were assessed for occlusive lesions at ×400 magnification. A vessel in which the lumen was partially (>50%) or fully obstructed was defined as an occlusive lesion (26, 37). Furthermore, all occlusive lesions were categorized as either cellular intimal thickening or intimal fibrosis. Cellular intimal thickening was identified by the characteristic, proliferating, intimal cellular masses that stained brown, whereas intimal fibrosis was identified by masses of less cellular fibrous tissue that stained bright red (13). Quantitative analysis was performed to determine both the proportion of occlusive lesions and the proportion of occlusive lesions with intimal fibrosis among all the small PAs per lung section (26, 37). The proportion of fibrotic area in the vessel area was determined by using a BIORÉVO BZ-9000 microscope (Keyence, Osaka, Japan) in Masson’s EVG-stained sections (7, 8). Fibrotic intimal area was defined as the area stained green inside of an external elastic lamina, and the vessel area

was determined as the area encircled by the external elastic lamina minus the area encircled by the luminal surface.

Morphometric analysis of medial wall thickness. The external diameters of small PAs in the lung section were measured along the shortest curvature (23, 24, 32, 33). The percent medial thickness of muscular arteries (OD 50–200 µm) was calculated with the formula [external diameter − internal diameter/external diameter] × 100 in EVG-stained slides (23, 24, 32).

Immunohistochemistry. Paraffin-embedded lung tissue slides were deparaffinized and hydrated. Epitope retrieval was performed by boiling the sections in citrate buffer (0.01 M, pH 6.0). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol. After blocking in 5% normal goat serum, mouse antibody to a cell proliferation marker Ki-67 (diluted 1:100) (clone MIB-5, Dako, Carpinteria, CA) or rabbit antibody to an apoptosis marker cleaved caspase-3 (diluted 1:50) (Asp175, Cell Signaling Technology, Beverly, MA) was applied to the sections. These sections were incubated with primary antibodies overnight at 4°C. After streptavidin-biotin amplification (LSAB2 kit, Dako), the slides were incubated with 3,3′-diaminobenzidine substrate and counterstained with hematoxylin (24, 32, 33).

Immunofluorescent staining. Paraffin-embedded lung tissue slides were incubated with primary antibodies for von Willebrand factor
(diluted 1:50, Millipore, Billerica, MA), cleaved caspase-3 (diluted 1:100, Cell Signaling), Ki67 (diluted 1:100, Dako), and survivin (diluted 1:400, Cell Signaling) overnight at 4°C, followed by incubation with Alexa 488 (goat anti-rabbit or anti-mouse)-conjugated secondary antibody (Molecular Probes, Eugene, OR). Next, the sections were stained with mouse anti-Cy3-conjugated smooth muscle actin (H9251-SMA) (diluted 1:100, Sigma) and TO-PRO-3 iodide (Molecular Probes) to visualize nuclei. Vessels were assessed by fluorescence microscopy. 

Fig. 4. Hemodynamic effects of macitentan in the early intervention study. Effects of macitentan on right ventricular systolic pressure (RVSP) (A), mean systemic arterial pressure (MSAP) (B), cardiac index (C), and the weight ratio of the right ventricle to the left ventricle + septum (RV/LV+S) (D). *P < 0.05 vs. Normal control. †P < 0.05 vs. SuHx 3 w + Vehicle 2 w. ‡P < 0.05 vs. SuHx 3 w. Values are means ± SE.

Fig. 5. Hemodynamic effects of macitentan in the late intervention study. Effects of macitentan on RVSP (A), MSAP (B), cardiac index (C), and RV/LV+S (D). *P < 0.05 vs. control rats. †P < 0.05 vs. SuHx 5 w + Vehicle 3 w. ‡P < 0.05 vs. SuHx 5 w. Values are means ± SE.
Cell proliferation and apoptosis assessment in lung tissue and other mechanistic studies. An immunohistochemical study for a cell proliferation marker, Ki67, and an apoptosis marker, cleaved caspase-3, was performed for the lung tissue sections. All occlusive lesions in vessels (OD, 15–50 μm) were classified into occlusive lesions with luminal monolayers positive or negative for Ki67 or cleaved caspase-3 and occlusive lesions with an underlying intima-media complex positive or negative for Ki67 or cleaved caspase-3. Quantitative analyses were performed by determining the proportion of occlusive lesions with an underlying intima-media complex positive for either marker in all occluded vessels. The proportion of occlusive lesions with luminal monolayers positive for either marker was also similarly determined. Immunofluorescent microscopic analyses of lung sections were performed to determine the proportion of occlusive lesions that included cleaved caspase-3+ or survivin+, α-SMA+ intimal-medial complex cells in occlusive pulmonary vascular lesions (OD, 15–50 μm) in each group. The proportion of occlusive lesions that included cleaved caspase-3+ or survivin+, α-SMA− luminal monolayer cells was also determined.

Quantitative RT-PCR. An RNeasy Mini Kit (Qiagen, Valencia, CA) was used to perform total RNA extraction from the peripheral lung tissue. A spectrophotometer was used to determine the final RNA amount. A cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) was used to perform reverse transcription PCR of 2.5 μg of RNA used as template. Quantitative RT-PCR was performed on a Step One Plus Real Time PCR System with TaqMan Gene Expression Assay on Demand probes. The following PCR primers were used: prepro-endothelin 1 (Rn00561129_m1), endothelin receptor type A

Fig. 6. Antiremodeling effects of macitentan in the early intervention study. Representative photographs (elastic van Gieson staining) of occlusive lesions (top; scale bar = 20 μm) and medial wall thickness of PAs (bottom; scale bar = 50 μm) (A) are shown. Effects of macitentan on percentage of vessels accompanied by occlusive lesions among the small PAs [outer diameter (OD), 15–50 μm] (B) and percentage of medial wall thickness in the small PAs (OD, 50–200 μm) (C) in the early intervention study. *P < 0.05 vs. Normal control. †P < 0.05 vs. SuHx 3 w + Vehicle 2 w. ‡P < 0.05 vs. SuHx 3 w. Values are means ± SE.
(Rn00561137_m1), endothelin receptor type B (Rn00569139_m1), bcl2 (Rn00437783_m1), Birc5 (survivin) (Rn00574012_m1), bax (Rn02532082_g1), and housekeeping gene HPRT1 (Rn01527840_m1) (Applied Biosystems, Foster City, CA). Step One software (Applied Biosystems) was used to determine relative standard curve values and values were normalized against HPRT1.

Western blot analysis. Lung tissue was homogenized in lysis buffer [150 mM NaCl, 0.5% Triton-X100, 50 mM Tris (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, phos STOP (Roche Applied Science), Complete EASY Pack (Roche)]. Thirty microgram of total protein, determined by using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL), was electrophoresed under reducing conditions by SDS-PAGE on 15% polyacrylamide gel (ePAGEL; Atto Chemicals, Tokyo, Japan) and transferred to a PVDF membrane by use of iBlot (Invitrogen, Carlsbad, CA). After blocking with PVDF Blocking Reagent (Toyobo, Osaka, Japan), membranes were incubated with primary antibodies, including anti-survivin (diluted 1:500, Cell Signaling), anti-cleaved caspase-3 (diluted 1:1,000, Cell Signaling), and anti-β-actin (diluted 1:2,000, Sigma-Aldrich, St. Louis, MO), at 4°C overnight. The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. A Western Lightning Plus-ECL chemiluminescence detection kit (Perkin-Elmer, Waltham, MA) was used to detect bands, which were quantified by use of an LAS3000 mini system and Multi-Gauge version 3.1 (Fuji Film, Tokyo, Japan).

Statistical analysis. GraphPad Prism 6 software (GraphPad Prism Software, San Diego, CA) was used to perform all analyses. One-way analysis of variance was used to compare variables among three or more groups, followed by Tukey’s multiple comparisons test. When two groups were compared, the unpaired two-tailed Student’s t-test was used to assess statistical differences. A P value of <0.05 was considered to be statistically significant. Data are presented as means ± standard errors of means.
RESULTS

All animals survived during the experimental period. Change in body weight and gene expression involved in endothelin-mediated pathway during the experimental period was described in Figs. 2 and 3.

Effects of macitentan on hemodynamic parameters. In the early intervention study, relative to the level in control rats (19.4 ± 1.4 mmHg), right ventricular systolic pressure (RVSP) increased in the 3-wk baseline PAH rats (78.3 ± 4.9 mmHg, $P < 0.05$) and PAH rats treated with vehicle for 2 wk (79.5 ± 6.4 mmHg, $P < 0.05$). RVSP was decreased, but not normalized, by macitentan treatment (50.3 ± 5.0 mmHg, vs. 3-wk baseline PAH rats, $P < 0.05$; vs. vehicle-treated PAH rats, $P < 0.05$) (Fig. 4A). Cardiac index was increased in macitentan-treated SuHx rats, relative to that in vehicle-treated PAH rats ($P < 0.05$), but not relative to that in control rats (Fig. 4C). These hemodynamic changes were consistent with the changes in the indexes of right ventricular hypertrophy (Fig.

![Image](image-url)

Fig. 8. Effects of macitentan on the development of intimal fibrosis in the late intervention study. Effects of macitentan on the percentage of vessels accompanied by intimal fibrosis in all the small PAs (OD, 15–50 μm) stained with elastic Van Gieson (EVG) (A and B) were evaluated. In addition, the proportion of fibrotic intimal area in the vessel area was determined by using Masson’s EVG in 30 randomly selected occlusive lesions per lung section. Such sections were obtained from 5 randomly selected rats in each group in the late intervention study (A and C). Representative photographs of occlusive lesions stained with EVG or Masson’s EVG are shown (A). *$P < 0.05$. Values are means ± SE.
In the late intervention study, RVSP increased in 5-wk baseline PAH rats (83.6 ± 7.5 mmHg vs. 23.6 ± 1.0 mmHg in control rats, P < 0.05) and in PAH rats treated with vehicle for 3 wk (82.5 ± 7.0 mmHg vs. normal controls, P < 0.05). RVSP was similarly decreased by macitentan treatment (53.3 ± 4.8 mmHg vs. 5-wk baseline PAH rats, P < 0.05; vs. vehicle-treated PAH rats, P < 0.05) (Fig. 5A). Cardiac index was comparable among the four treatment groups (Fig. 5C). Macitentan treatment attenuated right ventricular hypertrophy, relative to that in vehicle-treated PAH rats (P < 0.05), but not relative to that in 5-wk baseline PAH rats (P = 0.37) (Fig. 5D). The mean systemic arterial pressure was not modulated by the induction of PAH or macitentan treatment in either study (Figs. 4B and 5B).

Effects of macitentan on obstructive pulmonary vasculopathy. More than 200 small vessels (OD, 15–50 μm) per lung section were assessed in all sections. In the early intervention study, macitentan-treated PAH rats exhibited a lower proportion of occlusive lesions (17.9 ± 2.8%) compared with 3-wk baseline PAH rats (33.8 ± 4.4%, P < 0.05) and vehicle-treated PAH rats (41.5 ± 4.1%, P < 0.05) (Fig. 6, A and B). The % medial wall thickness (OD, 50–200 μm) in the macitentan group was also lower than in vehicle-treated PAH rats and 3-wk baseline PAH rats and was comparable to the control level (Fig. 6, A and C). In the late intervention study, macitentan treatment (30.1 ± 5.1%) decreased the proportion of occlusive lesions in PAH rats, relative to that in vehicle-treated PAH rats (50.0 ± 4.3%, P < 0.05), but not relative to that in 5 wk baseline PAH rats (40.8 ± 4.3%, P = 0.27) (Fig. 7, A and B). The % medial wall thickness in the macitentan group was lower than that in the vehicle-treated and 5-wk baseline PAH rats, but still higher than that in the control rats (Fig. 7, A and C).

Effect of macitentan on intimal fibrosis. The proportion of vessels with intimal fibrosis among small PAs was minimal in the 3-wk (data not shown) and 5-wk (0.5 ± 0.3%) PAH baseline rats. In the late intervention study, the proportion of vessels with intimal fibrosis was significantly increased in the PAH rats treated with vehicle for 3 wk (5.5 ± 2.1%, vs. 5-wk baseline PAH rats, P < 0.05). Macitentan treatment prevented an increase in vessels (%) with intimal fibrosis (0.5 ± 0.2% vs. vehicle-treated PAH rats, P < 0.05) (Fig. 8, A and B). These findings were further supported by indexes of an intimal fibrosis area determined by use of Masson’s elastic Van Gieson staining (Fig. 8, A and C).

Effects of macitentan on apoptosis and proliferation of cells in occlusive lesions. Von Willebrand factor+ luminal monolayers were supported by underlying intima-media complex cells positive for α-SMA in occlusive lesions (Fig. 9A). Cleaved caspase-3 and Ki67 were not obviously expressed in normal small PAs in control rats (Fig. 9B). In SuHx animals, expression of cleaved caspase-3 and Ki67 was confirmed in the α-SMA+ cells within the underlying intima-media complex as well as luminal monolayer cells by immunofluorescent confocal microscopic and/or immunohistochemical studies (Figs. 10, 11, and 12A). A total of 120–147 occlusive lesions in small PAs (OD, 15–50 μm) were assessed per lung section. In the early intervention study, 3-day macitentan treatment significantly increased cleaved caspase-3 expression and decreased Ki67 expression in the underlying intima-media complex cells in occlusive lesions (Fig. 10, A and C). In the late intervention study, in contrast, macitentan treatment exerted no effects on cleaved caspase-3 expression in such cells of occlusive lesions, although macitentan decreased the Ki67 expression (Fig. 11). These findings were further substantiated by quantitative immunofluorescent microscopic analyses for cleaved caspase-3+ cells in α-SMA+ intima-media complex cells or α-SMA− luminal cells in occlusive lesions (Fig. 12). However, 3-day treatment with macitentan did not exert effects on cleaved caspase-3 expression in whole lungs in the early or late studies (data not shown).

Effects of macitentan on survivin in occlusive lesions. Gene and protein expression of survivin was increased in the lungs of vehicle-treated PAH rats. Three-day macitentan treatment significantly decreased gene and protein expression of survivin in the early intervention study, but not in the late study (Fig. 13, A–D). Macitentan continued to suppress survivin mRNA expression until the end of the early intervention study but not of the late intervention study (Fig. 13, E and F). Furthermore, immunofluorescent confocal immunostaining showed that survivin was expressed in α-SMA+ intima-media complex cells and α-SMA− luminal monolayers in the occlusive lesions of vehicle-treated rats, but not in small PAs of control rats (Fig. 14, A–D). In α-SMA+ cells in the occlusive lesions, 3-day macitentan treatment suppressed the expression of survivin in

![](image1.png)

Fig. 9. Von Willebrand factor+ and α-smooth muscle actin+ cells in lungs of SuHx rats and cleaved caspase-3 and Ki67 expression in normal lungs. Representative photomicrographs of immunofluorescent confocal microscopic findings determined by use of antibodies for von Willebrand factor (VWF) and α-smooth muscle actin (α-SMA) are shown in occlusive lesions in Sugen/hypoxia-treated rats (A). Scale bar = 20 μm. TO-PRO-3, nuclear staining. Representative photomicrographs of immunohistochemical findings determined by using antibodies for cleaved caspase-3 and Ki67 in small PAs in control rats (B) (OD, 15–50 μm) are shown.
the early intervention study, but not in the late intervention study (Fig. 14, A and B), whereas no inhibition of survivin expression was found in luminal cells in either protocol (Fig. 14, E and F).

**DISCUSSION**

In the present study, compared with vehicle-treated PAH rats and baseline PAH rats evaluated before treatment initiation, macitentan-treated rats showed a decrease in the proportion of occlusive lesions in the early treatment study, consistent with the reversal of RVSP and the indexes of right ventricular hypertrophy and medial wall thickness; macitentan ameliorated but did not reverse the proportion of occlusive lesions in the late intervention study. Furthermore, macitentan increased the proportion of cleaved caspase-3+ apoptotic lesions and suppressed survivin expression in the early intervention study but not in the late intervention study whereas macitentan decreased the proportion of Ki67+ lesions at both stages of disease. Lastly, macitentan prevented the progression of fibrous changes in the intima, which was observed in the late intervention study. The present findings are summarized in a schematic diagram (Fig. 15).

**Histological reversal of occlusive pulmonary vasculopathy.** Evidence of the reverse remodeling effects of any intervention in PAH patients has been limited: such effects have been demonstrated only in PAH patients associated with congenital...
heart defects, in whom reversal of histological changes in PAH were shown by comparing the lung biopsy specimens obtained before and after PA banding (38). The present study demonstrated, for the first time, the histological reversal of established occlusive lesions by a clinically useful PAH compound to below the baseline level in this model, although prevention of the progression or amelioration of the vasculopathy, relative to that in vehicle-treated PAH animals using some agents, was previously described (4, 17, 19, 25, 37). Combined exposure to Sugen 5416 and chronic exposure with subsequent maintenance in ambient air up to 8 wk from the initial treatment reproduced severe pulmonary hypertension and right ventricular hypertrophy in the present experiment, with evidence of human PAH-like obstructive vascular disease and intimal fibrosis, as described previously (1, 13). The reverse remodeling effects of macitentan in this model were in agreement with the previously described reversing effects of other endothelin receptor antagonists in monocrotaline or hypoxic animal models, although the mechanisms involved in vivo were unclear in those studies (5, 6, 18, 40).

Mechanisms involved in the reverse remodeling effects of macitentan. The reversal of occlusive lesions in SuHx rats by macitentan in the early protocol was related to the consistent inhibition of proliferation and the induction of apoptosis in lumen-obstructing cells, which were associated with the inhibition of survivin expression in this model. The higher proportion of occlusive lesions observed in SuHx rats was characterized by the simultaneously increased expression of markers of proliferation and cell death in the present study, findings consistent with those of previous studies using a SuHx model (4, 17, 19, 25, 37). The in vivo roles of suppressing survivin and inducing caspase-3-mediated apoptosis in the reverse remodeling effects of macitentan in this model were consistent with those in vascular smooth muscle cells in previous reports involving monocrotaline rats and a vascular injury model, as well as those in human PAH (3, 22).

In the confocal microscopy study on SuHx rats 3–8 wk after Sugen treatment, lumen-obstructing cells primarily comprised /H9251-SMA/H11001 cells and luminal endothelial cells, similar to previously described results (1). Although both /H9251-SMA/H11001 cells and luminal endothelial cells, similar to previously described results (1). Although both /H9251-SMA/H11001 cells and luminal endothelial cells,
luminal endothelial cells were proliferative and apoptotic in this model, inhibition of lumen-obstructing cells by macitentan may have been primarily due to proapoptosis, at least in part, via survivin suppression and antiproliferation in α-SMA+ cells; macitentan inhibited the proliferation of luminal endothelial cells but did not affect the apoptosis of such cells. These findings were in agreement with the mitogenic and antiapoptotic effects of endothelin-1 in smooth muscle cells and myofibroblasts (6, 14, 16, 20) and with the roles of survivin in apoptosis resistance in cultured systemic and pulmonary vascular smooth muscle cells (3, 22), whereas endothelin-1 is also a mitogen for endothelial cells and promotes tubule formation.

Fig. 12. Effects of macitentan on cleaved caspase-3 expression in α-SMA+ intima-media complex or α-SMA− luminal monolayer cells in occlusive lesions. Effects of 3-day macitentan treatment on cleaved caspase-3 expression in α-SMA+ intima-media complex or α-SMA− luminal monolayer cells in occlusive lesions in the early (A, B, D) and late intervention studies (A, C, E). Representative photomicrographs of immunofluorescent confocal microscopic findings using antibodies for cleaved caspase-3 (green) and α-SMA (red) were shown in small PAs (OD, 15–50 μm) in each group (A). The proportion of occlusive lesions that included cleaved caspase-3+, α-SMA+ intima-media complex cells in 30 randomly chosen occlusive lesions (OD, 15–50 μm) per lung section is shown in the early (B) and late studies (C). The proportion of occlusive lesions that included cleaved caspase-3+, α-SMA− luminal monolayer cells in 30 randomly chosen occlusive lesions (OD, 15–50 μm) per lung section is also shown in the early (D) and late intervention studies (E). Such lung sections were obtained from all animals in each group. Scale bar = 20 μm. *P < 0.05. Values are means ± SE.
(31). Because the regulation of survivin expression in the setting of SuHx model may be complex and is poorly understood, the mechanism by which macitentan suppressed survivin expression is unclear. It is possible, however, that the blockade of the endothelin receptor signaling pathway directly inhibits survivin expression and subsequently induce caspase-3-mediated apoptosis in vascular cells, as reported in cell culture studies using myofibroblasts and fibroblasts (6, 14, 34). Thus the present findings support the hypothesis that endothelial dysfunction, represented by overproduction of endothelin-1, may be causally related to the persistence and/or progression of occlusive pulmonary vasculopathy through maintenance of survivin-mediated resistance to apoptosis and mitogenic properties in \( \alpha \)-SMA+ vascular cells in vivo.

Impact of the timing of treatment on physiological parameters and reverse remodeling effects. Consistent with the more prominent antiremodeling effects of macitentan on occlusive lesions in the early study relative to those in the late study, early treatment conferred distinct impact on other outcome parameters, including the reversal of right ventricular hypertrophy and normalization of medial thickening. Ostensibly similar decrease by macitentan in RVSP of SuHx rats in the early and late studies may be thereby related to the differential effects of macitentan on cardiac output in SuHx rats in the early treatment. However, the reversal of RVSP by macitentan in the presence of preserved cardiac output even in the late study could be explained by potential vasomotor effects of the compound (15) or reversal of medial thickening. Although an insignificant increase by macitentan in body weight change from initiating the experiment only in the early study may seem paradoxical, this finding could be explained by the combined effects of macitentan and withdrawal from hypoxia on body weight change during the treatment period. These findings therefore suggest that macitentan may have comparable systemic effects of macitentan in both intervention studies. Together, despite consistent time-dependent effects of ma-

![Fig. 13. Effects of macitentan on survivin protein and mRNA expression in the whole lungs. Effects of 3-day macitentan treatment on mRNA and protein expression of survivin in the whole lungs in the early (A and C) and late intervention studies (B and D). Effects of macitentan treatment on mRNA expression of survivin in the lungs at the end of the early (E) and late intervention studies (F) are also shown. *P < 0.05. Values are means ± SE.](http://ajplung.physiology.org/)

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**A**

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<tr>
<th>Group</th>
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**F**

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Fig. 14. Effects of macitentan on survivin expression in α-SMA+ intima-media complex or α-SMA− luminal monolayer cells in occlusive lesions. Representative pictures of survivin expression in α-SMA+ intima-media complex or α-SMA− luminal monolayer cells in occlusive lesions (OD, 15–50 μm) were shown in 3-day vehicle- or macitentan-treated PAH rats in the early (A) and late (B) studies. The proportion of occlusive lesions that included survivin+, α-SMA− luminal-media cells in 30 randomly chosen occlusive lesions (OD, 15–50 μm) per lung section is shown in the early (A) and late intervention studies (B). Representative photomicrographs of immunofluorescent confocal microscopic findings determined by using antibodies for α-SMA and survivin were shown in small PAs in control rats (C). Representative pictures of survivin expression in α-SMA− luminal monolayers of occlusive lesions were also shown in vehicle- and macitentan-treated PAH rats (D). The proportion of occlusive lesions that included survivin+, α-SMA− luminal monolayer cells in 30 randomly chosen occlusive lesions (OD, 15–50 μm) per lung section is shown in the early (E) and late intervention studies (F). Lung sections were from all animals in each group. Scale bar = 20 μm. *P < 0.05. Values are means ± SE.
citation on occlusive vasculopathy in SuHx rats, vasomotor effects or pulmonary vascular diseases other than occlusive vasculopathy may have a role in the global impact of the compound in PAH in animals or patients (27).

Mechanisms involved in the time-dependent effects of macitentan on survivin-mediated resistance to apoptosis are immediately unknown. Since the level of an increase in survivin expression at the end of the late study (SuHx 5 w+ Vehicle 3 w) seems to be less prominent, it is possible that the abnormal induction of survivin and apoptosis resistance is a more relevant mechanism in early stages of the disease and at later stages additional mechanisms dictate disease progression. Alternatively, since survivin, if any, is still upregulated in the SuHx rats at such a later time point, the ability of macitentan to repress survivin expression in α-SMA+ cells is time limited. It could be related to the altered expression of endothelin receptors in SuHx rats, in which endothelin receptor A mRNA was upregulated in the early intervention study, but not in the late intervention study; endothelin receptor B mRNA was rather downregulated only in the late intervention study.

Fibrotic change in occlusive lesions, which was targeted by macitentan in the late intervention study, have only recently been appreciated in this model (1); the antifibrotic effects of any compounds have not been investigated in this model. Because there was no intimal fibrosis in monocrotaline or hypoxia models, this is the first investigation involving the mechanisms involved in antiremodeling effects of a PAH compound in a later and more intractable stage of the same PAH model, which could be mechanistic basis for the efficacy of early treatment and gives an insight into further appearance of resistance to treatment for this disorder. Because the early detection of PAH remains a challenging issue with significant limitations in the clinical setting (9), future studies are warranted to verify the mechanisms involved in the resistance to apoptosis and the fibrotic changes in the advanced occlusive vasculopathy in PAH.

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DISCLOSURES
Y. Mitani declares that macitentan was gifted from Actelion Pharmaceuticals, Allschwil, Switzerland.

AUTHOR CONTRIBUTIONS

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