Effect of All-Trans-Retinoic Acid on the Development of Chronic Hypoxia-Induced Pulmonary Hypertension

Erquan Zhang, MD; Baohua Jiang, MD; Ayumu Yokochi, MD; Junko Maruyama, MD;
Yoshihide Mitani, MD; Ning Ma, MD; Kazuo Maruyama, MD

Background: An earlier study showed that all-trans-retinoic acid (ATRA) prevents the development of mono-crotalin-induced pulmonary hypertension (PH). The purpose of the present study was to determine the effect of ATRA on another model of chronic hypoxia-induced PH.

Methods and Results: Male Sprague−Dawley rats were given 30 mg/kg ATRA or vehicle only by gavage once daily for 14 days during hypobaric hypoxic exposure. Chronic hypoxic exposure induced PH, right ventricular hypertrophy (RVH), and hypertensive pulmonary vascular changes. Quantitative morphometry of the pulmonary arteries showed that ATRA treatment significantly reduced the percentage of muscularized arteries in peripheral pulmonary arteries only with an external diameter between 15 and 50 μm. ATRA treatment also significantly reduced the medial wall thickness in small muscular arteries only with an external diameter between 50 and 100 μm. Unfortunately, these reductions did not accompany the lowering of pulmonary artery pressure nor decrease in RVH. Chronic hypoxia-induced PH rats with ATRA had a loss in body weight. Chronic hypoxia increased the expression of endothelial nitric oxide synthase in the lung on western blotting and immunohisto-chemistry, in which ATRA treatment had no effect.

Conclusions: The administration of ATRA might not have a therapeutic role in preventing the development of chronic hypoxia-induced PH, because of body weight loss and the subtle preventable effects of vascular changes. (*Circ J* 2010; **74:** 1696–1703)

Key Words: Hypoxia; Nitric oxide; Pulmonary hypertension; Retinoic acid

U nder all conditions causing pulmonary hypertension (PH), vascular changes include the new muscular-ization of normally non-muscular peripheral pulmo-nary arteries and medial hypertrophy of muscular arteries.[1–8] Plasma levels of all-trans-retinoic acid (ATRA) and 13-cis retinoic acid are reduced in patients with idiopathic PH compared to control subjects.[9] ATRA treatment ameliorated the rise in pulmonary artery pressure (PAP) in monocrotaline-induced PH in rats.[10] Some of the steps to progressive fibro-sis in monocrotaline-induced PH could be interrupted by retinoic acid treatment.[11] According to these studies, ATRA treatment might also ameliorate the development of another model of PH, that is, chronic hypoxia-induced PH.

Retinoic acid reduces the proliferation of cultured vascu-lar smooth muscle cells in vitro.[12] Platelet-derived growth factor-BB and serum-stimulated rat aortic smooth muscle cell DNA syntheses and cell proliferation in culture were also inhibited by retinoic acid.[13] In addition, supplemental retinoid reduces neointimal formation in the rat carotid artery after balloon withdrawal injury.[14] A recent study showed that ATRA increases nitric oxide (NO) synthesis by endothelial cells.[15] The upregulation of NO synthase (NOS) by ATRA has been shown in cultured vascular endothelial cells[16] and coronary arteries in chronic kidney disease.[17] We previously showed that the NO precursor L-arginine reduced the devel-opment of PH and vascular remodeling in monocrotaline-induced PH and chronic hypoxia-induced PH rats.[2] Continu-ously inhaled NO also prevented the development of PH in the latter rats.[3] Thus, retinoic acid might reduce the devel-opment of PH directly through cell growth inhibition or indi-rectly through the production of NO. Therefore, we designed this study to determine if ATRA prevents the development of PH and hypertensive pulmonary vascular changes in chronic hypoxia-induced PH in rats.

Methods

The Animal Experiment Committee of Mie University School

of Medicine approved the study protocol. Six-week-old male Sprague–Dawley rats (Clea, Japan) weighing 180–300 g were used. Each animal was randomly assigned to one of 5 groups: (1) rats exposed to ambient air with no treatment (Air/None, n=11); (2) rats exposed to ambient air and gavaged with corn oil (Air/Oil, n=11); (3) rats exposed to ambient air and gavaged with ATRA, (Air/ATRA, n=10); (4) rats exposed to hypoxia for 14 days (air at 380 mmHg) and gavaged with corn oil (PH/Oil, n=13); and (5) rats exposed to hypobaric hypoxia and gavaged with ATRA (PH/ATRA, n=15). The animals gavaged with ATRA received a once daily dose of 30 mg of ATRA (Sigma) suspended in 1.5 ml of corn oil. Fresh suspensions of ATRA were prepared each day while avoiding light exposure as much as possible. Gavage was started from 1 day before hypoxic exposure and continued for 14 days, finishing on the morning of the 14th day after the hypoxic exposure. Food and water were provided ad libitum. According to previous studies,[4,6] the rats were removed from the hypobaric chamber once daily for 30 min for gavage. Body weight was measured every day.

Structural Assessment

Preparation of Lung Tissue for Morphometry Fourteen days after the hypoxic exposure, rats were anesthetized with pentobarbital sodium (45 mg/kg) administered i.p. Blood samples were collected for red cell counts, hematocrit measurement, analytical chemistry, and plasma nitrate level measurement. A lung section for morphometric analysis of the vasculature was prepared using the barium injection method, which has been reported in detail.[5,6] Briefly, the pulmonary artery was injected with a hot radiopaque barium-gelatin mixture at 100 cmH2O pressure. After injection, the lung was distended and perfused through the tracheal tube with 10% formalin at 36 cmH2O pressure for 72 h. Sections were stained for elastin by the Van Gieson method. The right ventricle (RV) of the heart was dissected from the left ventricle plus septum (LV+S) and weighed separately. The heart weight ratio (RV/LV+S) was calculated to assess right ventricular hypertrophy (RVH).

Morphometry of Pulmonary Arteries Light microscope slides were analyzed, without previous knowledge of the treatment groups. All barium-filled arteries in each tissue section were examined at ×400, for an average of 349 arteries per section (200–596 arteries per section). Each artery was identified as being one of two structural types for the presence of muscularity: muscularized (with a complete medial coat, incomplete medial coat, or only a crescent of muscle being present), and non-muscular (no muscle apparent).[2,4–8] The percentage of muscularized arteries in peripheral pulmonary arteries with an external diameter between 15 μm and 50 μm and between 50 μm and 100 μm was calculated. For muscular arteries between 50 μm and 100 μm in diameter and those between 100 μm and 200 μm in diameter (an average of 18 arteries [4–49 arteries] were found per section), the wall thickness of the media (distance between external and internal elastic laminae) was measured along the shortest curvature, and the percent medial wall thickness (%MWT) was calculated.[2,4–8]

PAP

In another set of experiments, 26 rats were used to test the effect of the prolonged administration of ATRA on the mean PAP during the development of chronic hypoxia-induced PH. Rats were randomly assigned to 1 of 4 groups: rats exposed to ambient air and gavaged with corn oil (Air/Oil, n=8), rats

exposed to ambient air and gavaged with ATRA (Air/ATRA, n=6), rats exposed to hypobaric hypoxia for 14 days and treated with vehicle (PH/Oil, n=6), and rats exposed to hypobaric hypoxia for 14 days and treated with ATRA (PH/ATRA, n=6). The administration of ATRA (30 mg/kg, daily) began 1 day before the hypoxic exposure period and continued until the end of the 14 days of hypoxic exposure. After the hypoxic exposure period, a pulmonary artery catheter (Silastic tubing, 0.31 mm inner diameter and 0.64 mm outer diameter) was inserted via the right external jugular vein into the pulmonary artery using a closed-chest technique, as previously described.[2,4–8] Forty-eight hours after catheterization with the rat fully conscious, allowing sufficient time for recovery from the effect of anesthesia, the PAP was recorded. The mean PAP was recorded with a physiological transducer and an amplifier system (AP 620G, Nihon Kohden, Japan) once the rats were calm.

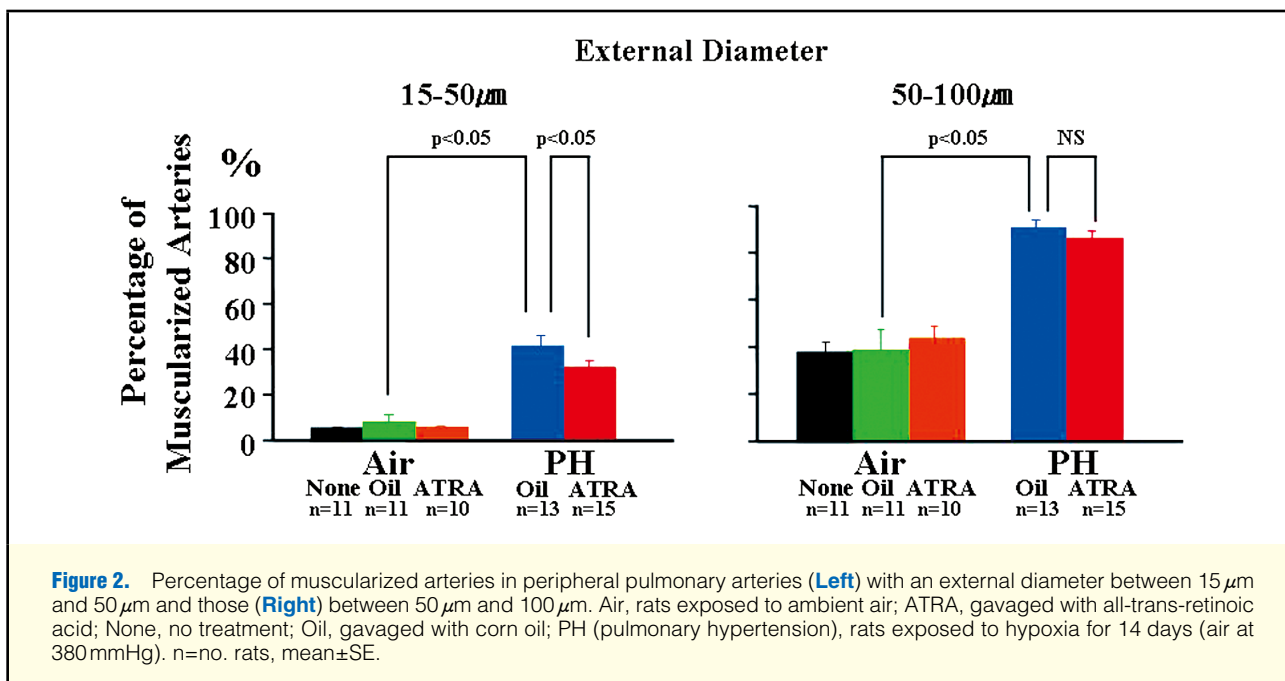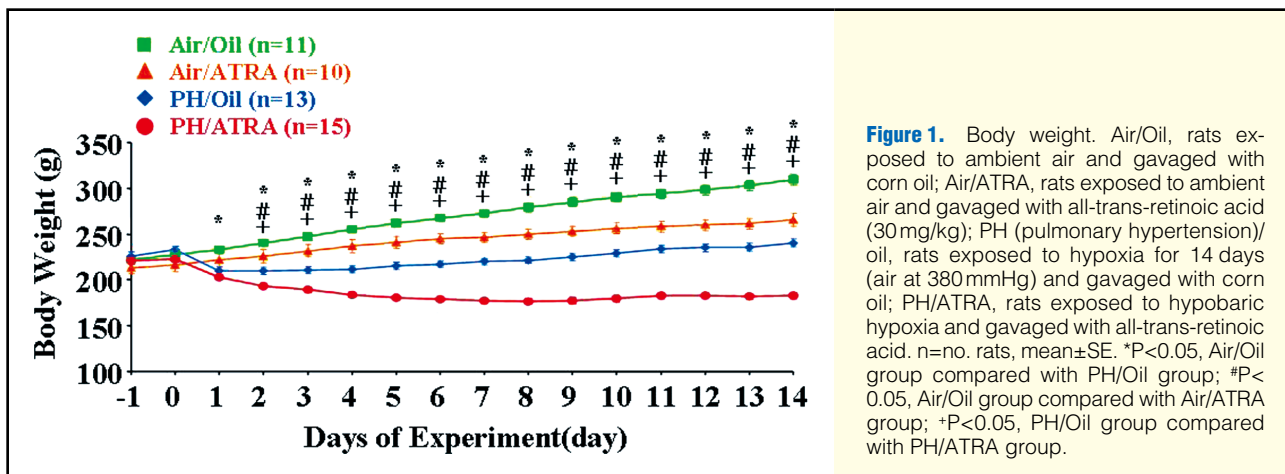## Measurement of Plasma Retinoic Acid and Nitrate

To confirm the effectiveness of retinoic acid supplementation, we determined the plasma ATRA level. Blood samples were obtained from rats 2 h after gavage of ATRA (30 mg/kg) or vehicle under reduced lighting conditions. The ATRA levels were determined on high-performance liquid chromatography with a high-pressure cadmium column (LC-10A, Shimazu, Japan). The detection limit was 0.5 ng/ml retinoic acid. Plasma samples for nitrate were stored at –20°C until assay.

Western Blotting for Endothelial NOS (eNOS)

In another set of experiments with 13 rats, western blotting for eNOS in whole lung tissue was performed in rats exposed to hypoxia with and without ATRA for 10 days. After death by decapitation and exsanguination under pentobarbital anesthesia, lung specimens were obtained. Samples were homogenized and centrifuged, and the supernatant was subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis on 10% acrylamide gels and blotted onto a polyvinylidene difluoride membrane. Blots were incubated with a mouse monoclonal antibody raised against mouse eNOS. Primary antibody (BD Transduction Laboratories G10296, 1:2,500 dilution) and a secondary antibody (Amersham NA 931, 1:20,000 dilution) were used. In addition to samples, molecular weight standards, eNOS standards (mouse macrophage; Transduction Laboratories), and beta actin (Sigma A 5441, 1:200,000 dilution) were run at the same time.

Immunofluorescence

Each group of rats (Air/None, n=3; PH/Oil, n=3; PH/ATRA, n=3) was deeply anesthetized with an i.p. injection of sodium pentobarbital and were perfused transcardially with fixative that contained 4% formalin in 0.01-mol/L phosphate-buffered saline. After the perfusion, lung was dissected out and allowed to stand in the same fixative for several hours. Then they were rinsed several times with 0.01-mol/L phosphate-buffered saline, dehydrated with a graded alcohol series and acetone, and embedded in paraffin. Six-μm-thick sections were mounted on silanized slides. Sections were deparaffinized in xylene, rehydrated in graded alcohol solutions. The lung sections were further subjected to antigen retrieval by microwave in 5% urea buffer for 5 min and slowly cooled to room temperature. After blocking of non-specific sites with 1% skim milk in 0.01-mol/L phosphate-buffered saline for 30 min, sections were incubated overnight (4°C) with monoclonal antibody against eNOS (1:300, catalog no. 610297,

Figure 1. Body weight. Air/Oil, rats exposed to ambient air and gavaged with corn oil; Air/ATRA, rats exposed to ambient air and gavaged with all-trans-retinoic acid (30 mg/kg); PH (pulmonary hypertension)/oil, rats exposed to hypoxia for 14 days (air at 380 mmHg) and gavaged with corn oil; PH/ATRA, rats exposed to hypobaric hypoxia and gavaged with all-trans-retinoic acid. n=no. rats, mean±SE. *P<0.05, Air/Oil group compared with PH/Oil group; #P<0.05, Air/Oil group compared with Air/ATRA group; +P<0.05, PH/Oil group compared with PH/ATRA group.

Figure 2. Percentage of muscularized arteries in peripheral pulmonary arteries (Left) with an external diameter between $15\,\mu m$ and $50\,\mu m$ and those (Right) between $50\,\mu m$ and $100\,\mu m$. Air, rats exposed to ambient air; ATRA, gavaged with all-trans-retinoic acid; None, no treatment; Oil, gavaged with corn oil; PH (pulmonary hypertension), rats exposed to hypoxia for 14 days (air at 380 mmHg). n=no. rats, mean±SE.

BD Transduction Laboratories) and treated with goat anti-mouse IgG-Alexa594 (1:500, catalog no. A11032, Invitrogen) for 2 h at room temperature. The stained sections were examined under an inverted Laser Scan Microscope (Fluoview FV1000, Olympus, Tokyo, Japan). To identify the immune-negative structures and endothelial cells of blood vessel in the lung, some neighboring sections were stained with hematoxylin and eosin.

Data Analysis

Data are expressed as mean±SE. When more than two means were compared, one-way analysis of variance was used. When significant variance was found, Fisher's protected least significant difference test was used to establish which groups were different. Differences were considered significant at P<0.05.

Results

Body Weight (Figure 1)

Initial body weight was similar in all groups. PH/Oil rats lost weight during the first several days of hypoxic exposure, but regained it afterward. In contrast, the rats kept in ambient air (air rats) gained weight steadily. From day 1 through the last day, PH/Oil rats had significantly lower body weight than Air/Oil rats. ATRA treatment significantly reduced the body weight in both Air/ATRA and PH/ATRA rats compared to Air/Oil and PH/ATRA rats, respectively.

Vascular Structural Changes

The percentage of muscularized arteries was similar between Air/None and Air/Oil rats. Hypoxia significantly increased the percentage of muscularized arteries in peripheral pulmonary arteries from 8.0±2.4% to 41.2±5.0% (P<0.05), in peripheral pulmonary arteries with external diameters between $15\,\mu m$ and $50\,\mu m$ and from 38.7±8.9% to 90.8±2.8% (P<0.05) in peripheral pulmonary arteries with external diameters between $50\,\mu m$ and $100\,\mu m$ (Figure 2). ATRA significantly reduced this increase in the percentage of muscularized arteries in peripheral pulmonary arteries with an external diameter between $15\,\mu m$ and $50\,\mu m$, but not in those between $50\,\mu m$ and
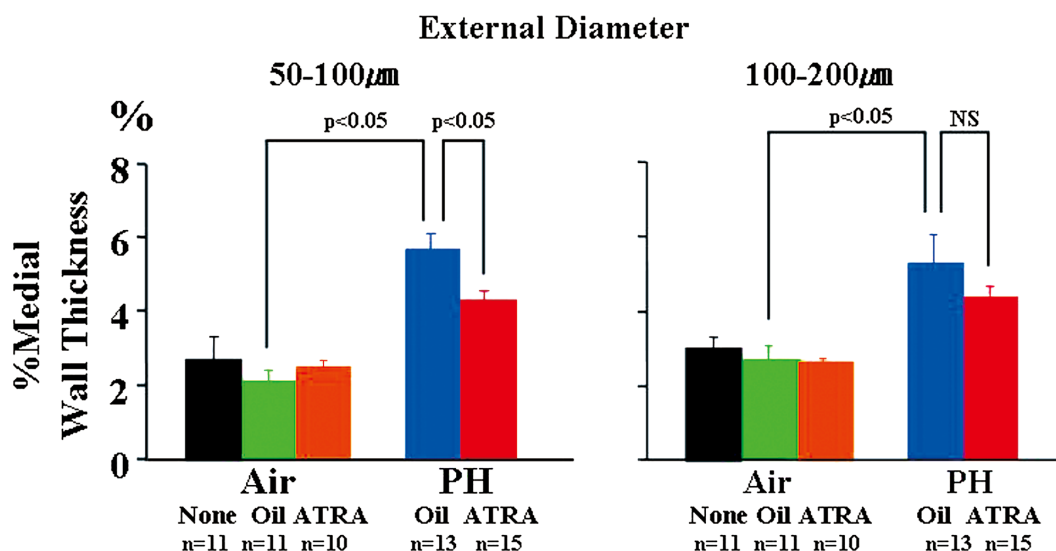
Figure 3. % medial wall thickness in small muscular arteries (**Left**) between 50 μm and 100 μm in diameter and (**Right**) those between 100 μm and 200 μm in diameter. Air, rats exposed to ambient air; ATRA, gavaged with all-trans-retinoic acid; None, no treatment; Oil, gavaged with corn oil; PH (pulmonary hypertension), rats exposed to hypoxia for 14 days (air at 380 mmHg). n=no. rats; mean±SE.

Table 1. Blood Chemistry

	Air			PH	
	None (n=11)	Oil (n=11)	ATRA (n=10)	Oil (n=13)	ATRA (n=15)
Alb (g/dl)	2.8±0.3	3.1±0.2	2.8±0.2	4.0±0.1*	2.9±0.1+
BUN (mg/dl)	13.4±1.6	14.4±0.8	16.4±1.3	16.9±0.7	18.5±0.8
Creatinine (mg/dl)	0.22±0.03	0.22±0.02	0.24±0.02	0.30±0.01*	0.25±0.01+
AST (IU/L)	77.5±9.0	92.0±7.2	111.8±7.4	129.7±8.8*	152.1±12.1
ALT (IU/L)	36.9±5.0	38.2±1.6	47.5±3.4	55.4±6.8	62.2±8.5
ALP (IU/L)	726±83	827±109	1,250±118#	1,231±74*	1,326±65
Ch-E (IU/L)	8.9±4.5	5.1±0.6	5.8±0.9	8.1±0.6	7.4±0.6
Triglyceride (mg/dl)	60.9±7.6	87.7±12.6	219.5±40.3#	68.4±11.7	98.7±13.0
Glu (mg/dl)	160±21	190±13	191±12	182±10	202±12

Data are given as mean±SE. #$P<0.05$, Air/Oil group compared with Air/ATRA group; *$P<0.05$, Air/Oil group compared with PH/Oil group; +$P<0.05$, PH/Oil group compared with PH/ATRA group.
Air, rats exposed to ambient air; PH (pulmonary hypertension), rats exposed to hypoxia for 14 days (air at 380 mmHg); None, no treatment; Oil, gavaged with corn oil; ATRA, gavaged with all-trans-retinoic acid; Alb, albumin; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Ch-E, cholinesterase; Glu, glucose.

100 μm.

The %MWT was similar between Air/None and Air/Oil rats. Hypoxia significantly increased the %MWT from 2.64± 0.43% to 3.29±0.76% in the small muscular arteries between 50 μm and 100 μm in external diameter (P<0.05), and also from 2.08±0.31% to 5.66±0.39% in those between 100 μm and 200 μm in external diameter (P<0.05) usually accompanied by a terminal or respiratory bronchiolus (**Figure 3**). ATRA significantly reduced this increase in the %MWT induced by hypoxia in muscular arteries with an external diameter between 50 μm and 100 μm (P<0.05), but not in those with an external diameter between 100 μm and 200 μm.

Blood Chemistry and Red Cell Count
Table 1 summarizes the results of blood chemistry measurements. There were no differences in each item of blood

chemistry between Air/None and Air/Oil rats. ATRA treatment produced significant elevations in triglyceride, alanine aminotransferase (ALT), serum alkaline phosphatase, and creatinine kinase levels in Air/ATRA compared to Air/Oil rats. Hypoxia also produced significant elevations in ALT, serum alkaline phosphatase, and creatinine kinase. ATRA treatment had no effect on triglyceride, ALT, serum alkaline phosphatase, and creatinine kinase levels in hypoxic PH rats (**Table 1**). ATRA treatment significantly reduced the red cell count, hemoglobin concentration, and hematocrit in air rats. Hypoxia increased the red cell count, hemoglobin concentration, and hematocrit. ATRA treatment significantly reduced these increases induced by hypoxic exposure (**Table 2**).

Mean PAP (mPAP) and RVH
The mean PAP of the Air/Oil and Air/ATRA rats were simi-

Table 2. RBC and Platelet Count

	Air			PH	
	None (n=11)	Oil (n=11)	ATRA (n=10)	Oil (n=13)	ATRA (n=15)
RBC (10^6/μl)	7.82±0.14	7.91±0.15	7.35±0.12#	10.54±0.14*	9.49±0.19+
Hemoglobin (g/dl)	14.2±0.2	14.3±0.2	13.2±0.3#	19.5±0.2*	16.9±0.2+
Hematocrit (%)	43.4±0.6	43.8±0.6	40.1±0.7#	60.8±0.7*	52.6±0.8+
Platelets (10^3/μl)	1,031±80	1,196±49	1,077±93	890±62*	959±74

Data are given as mean±SE. #$P<0.05$, Air/Oil group compared with Air/ATRA group; *$P<0.05$, Air/Oil group compared with PH/Oil group; +$P<0.05$, PH/Oil group compared with PH/ATRA group.
RBC, red blood cell. Other abbreviations see in Table 1.
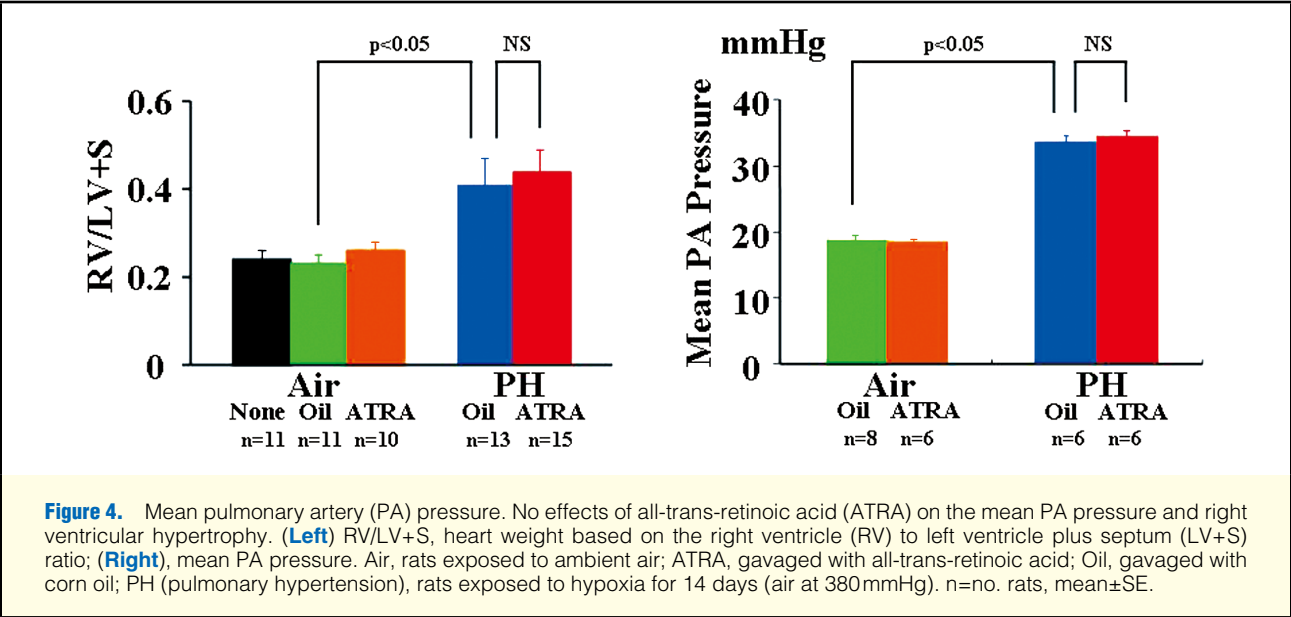
Figure 4. Mean pulmonary artery (PA) pressure. No effects of all-trans-retinoic acid (ATRA) on the mean PA pressure and right ventricular hypertrophy. (**Left**) RV/LV+S, heart weight based on the right ventricle (RV) to left ventricle plus septum (LV+S) ratio; (**Right**), mean PA pressure. Air, rats exposed to ambient air; ATRA, gavaged with all-trans-retinoic acid; Oil, gavaged with corn oil; PH (pulmonary hypertension), rats exposed to hypoxia for 14 days (air at 380 mmHg). n=no. rats, mean±SE.
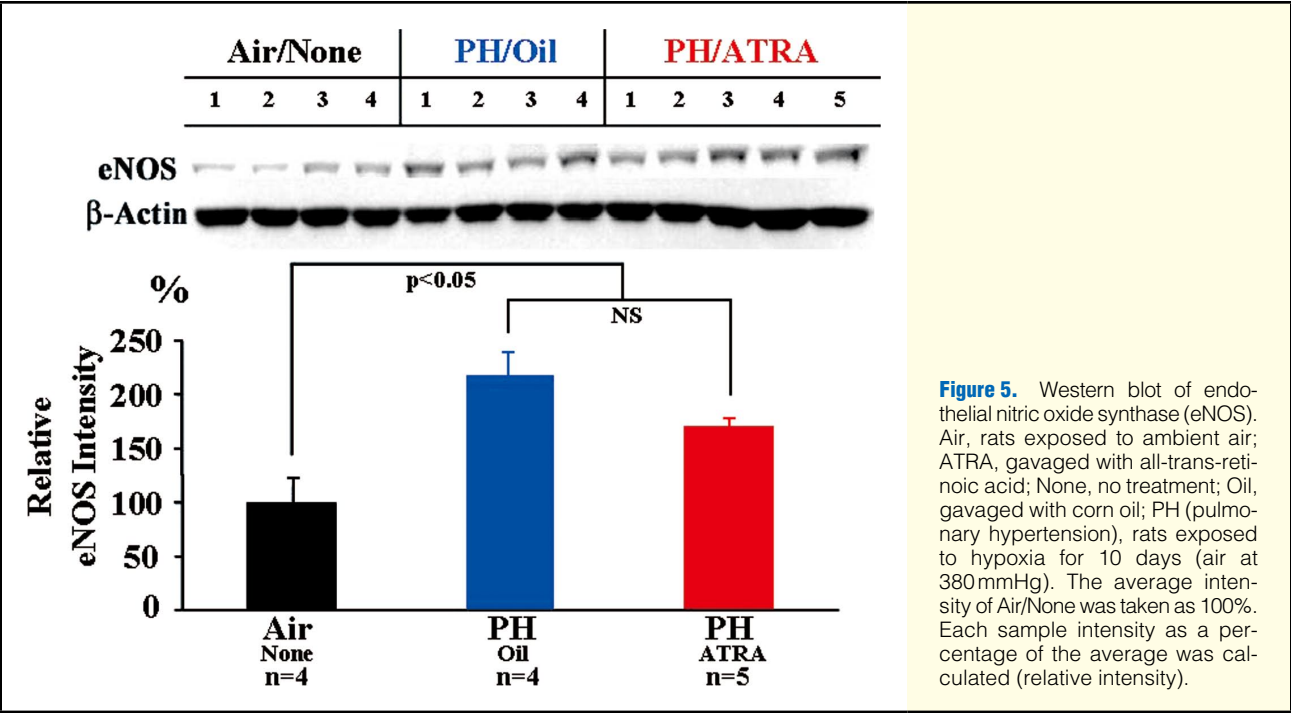
Figure 5. Western blot of endothelial nitric oxide synthase (eNOS). Air, rats exposed to ambient air; ATRA, gavaged with all-trans-retinoic acid; None, no treatment; Oil, gavaged with corn oil; PH (pulmonary hypertension), rats exposed to hypoxia for 10 days (air at 380 mmHg). The average intensity of Air/None was taken as 100%. Each sample intensity as a percentage of the average was calculated (relative intensity).
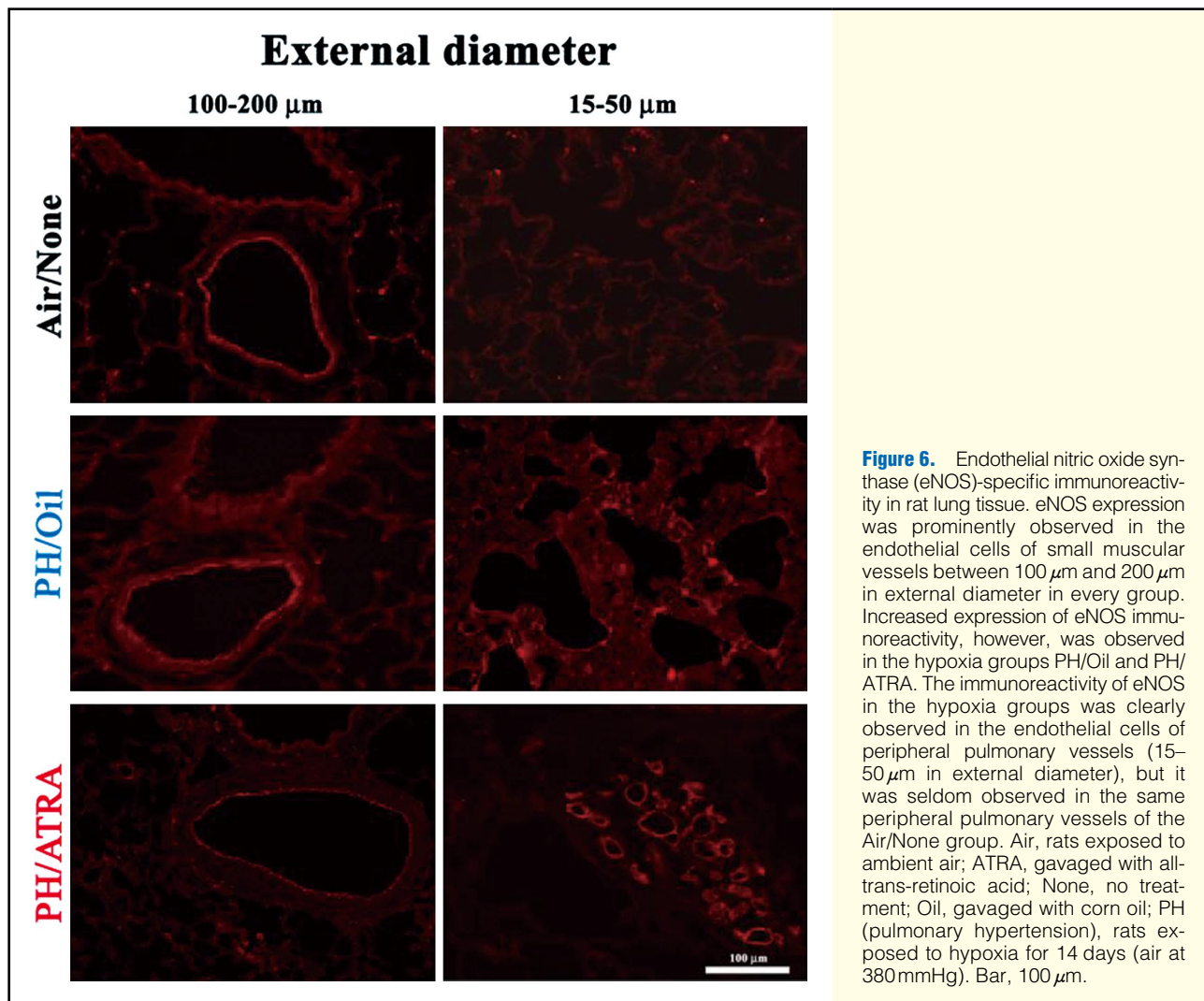
Figure 6. Endothelial nitric oxide synthase (eNOS)-specific immunoreactivity in rat lung tissue. eNOS expression was prominently observed in the endothelial cells of small muscular vessels between 100 μm and 200 μm in external diameter in every group. Increased expression of eNOS immunoreactivity, however, was observed in the hypoxia groups PH/Oil and PH/ATRA. The immunoreactivity of eNOS in the hypoxia groups was clearly observed in the endothelial cells of peripheral pulmonary vessels (15–50 μm in external diameter), but it was seldom observed in the same peripheral pulmonary vessels of the Air/None group. Air, rats exposed to ambient air; ATRA, gavaged with all-trans-retinoic acid; None, no treatment; Oil, gavaged with corn oil; PH (pulmonary hypertension), rats exposed to hypoxia for 14 days (air at 380 mmHg). Bar, 100 μm.

lar. Chronic hypoxia increased mPAP and RV/LV+S compared with air rats. The prolonged administration of ATRA had no effect on mPAP and RV/LV+S in chronic hypoxia-induced PH rats (**Figure 4**).

Plasma ATRA and Nitrate Concentration
ATRA concentration was 0.65 ng/ml (n=2) in vehicle-administered rats and 2,011.3±409.3 ng/ml (n=4) in rats gavaged with ATRA, suggesting the effectiveness of ATRA supplementation by oral gavage. Plasma nitrate concentration was 11.4±0.75 mmol/L in control rats (n=5), 15.8±1.39 mmol/L (n=5; P<0.05, compared to control) in rats exposed to hypobaric hypoxia without ATRA, and 15.4±1.12 mmol/L (n=5; NS, compared to rats without ATRA) exposed to hypobaric hypoxia and treated with ATRA.

Western Blotting for eNOS
Protein expression of eNOS was significantly upregulated in the lungs of rats exposed to the 10 days of hypobaric hypoxia. ATRA had no effect on this upregulation (**Figure 5**).

eNOS Expression in Rat Lung
Figure 6 shows the distribution of eNOS-specific immunoreactivity in the lung tissue. eNOS expression was promi-

nently observed in the endothelial cells of small muscular vessels between 100 μm and 200 μm in external diameter in every group. The immunoreactivity of eNOS in the hypoxia groups (PH/Oil, PH/ATRA) was clearly observed in the endothelial cells of peripheral pulmonary vessels (15–50 μm in external diameter), but it was seldom observed in the same peripheral pulmonary vessels of the Air/None group.

Discussion
Although ATRA significantly decreased the percentage of muscularized arteries in peripheral pulmonary arteries (diameter 15–50 μm), this parameter was still much greater than in any of the control animals. Likewise, although ATRA significantly decreased the %MWT in arteries with a diameter of 50–100 μm, the %MWT remained much greater than in any of the control animals. Thus, although there were statistically significant decreases in these two anatomic parameters, whether there was concurrent physiologic significance is questionable. The lack of improvement in PH or RVH suggests that this is the case.

The dose regimen in the present study (30 mg·kg⁻¹·day⁻¹) was the same as in the Miano et al study, in which ATRA administration led to a reduction in neointimal formation in

the rat carotid artery after balloon withdrawal injury.[14] Qin et al. also used the same dose, in which ATRA partly prevented the development of PH in monocrotaline-injected rats.[10] This dose is higher than that administered to humans undergoing induction therapy for neoplastic disease. Earlier studies showed that ATRA treatment and chronic hypoxic exposure led to mild growth retardation, respectively.[18,19] Combined hypoxic exposure and ATRA treatment led to marked growth retardation, which might be a major side-effect, especially in rats during hypoxic exposure. Because of this growth retardation during hypoxic exposure, the dose escalation of ATRA seemed unreasonable. Hypertriglyceridemia and elevated alkaline phosphatase in ATRA-treated rats in ambient air are consistent with earlier studies.[18–20] The inhibitory effect of ATRA on erythropoiesis might be strengthened under hypoxic conditions, because several toxicological studies have shown that ATRA induces anemia in rats.[19–21] Although polycythemia might contribute to the increase in PAP, the effect of reduced degree of polycythemia by ATRA seemed to be minimal because no difference in PAP was observed in hypoxic rats with or without ATRA. The 30-min air-breathing period for gavage each day probably had little effect in inducing PH, because intermittent hypoxic exposure of rats to 8–10.5% oxygen for between 4 and 8 h per day has been shown to cause PH, RVH and pulmonary vascular remodeling.[22]

A recent study showed that ATRA reduced the rise in PAP in monocrotaline-induced PH in rats.[10] Several possibilities should be mentioned as to why ATRA is effective for monocrotaline-induced PH but not for chronic hypoxia-induced PH. First, inflammatory response is marked in the monocrotaline model but not in the hypoxia-induced model. Dietary retinol prevents inflammatory responses such as mononuclear inflammatory cell infiltration into alveolar septa or the medial wall of the pulmonary artery in monocrotaline-induced PH.[23] The monocrotaline-induced elevation of tumor necrosis factor-α, interferon-γ, matrix metalloproteinase-1 in lung is depressed by ATRA.[10,11] Inflammatory response is subtle, if any, in chronic hypoxia-induced PH compared to the monocrotaline model, suggesting few inflammation-related targets for ATRA. Inhibition of inducible NOS did not ameliorate hypoxia-induced PH.[24] Second, the role of the vasoconstrictor component may be greater in the hypoxia-induced model than in the monocrotaline model. The pressure rise induced by hypoxic pulmonary vasoconstriction is the initial response in chronic hypoxia-induced PH, which is followed by hypertensive vascular remodeling.[25] In contrast, in the monocrotaline model pulmonary vascular endothelial changes and subsequent vascular remodeling precede the rise in PAP.[26] There is also evidence that Rho-kinase-mediated vasoconstriction contributes substantially to the increased pulmonary arterial pressure in the monocrotaline model.[27,28] Hypoxia activates Rho-kinase in vascular smooth muscle and induces vasoconstriction.[29] In patients with PH, Rho-kinase activity is increased.[30] There is accumulating evidence that acutely reversible vasoconstriction but not fixed vascular remodeling plays an essential role in the pathogenesis of chronic hypoxia-induced PH in rats. For instance, Nagaoka et al and Hyvelin et al have demonstrated that iv Rho-kinase inhibitor acutely normalizes the PH in chronically hypoxic rats.[31,32] Furthermore, Crossno et al have shown that the PPAR-γ agonist rosiglitazone inhibits pulmonary vascular remodeling but fails to block the development of chronic hypoxia-induced PH because of inability to reverse sustained Rho-kinase-mediated vasoconstriction.[33] Thus there is the possibility that ATRA did not prevent the development of chronic hypoxia-induced PH because it does not have any effect on sustained vasoconstriction. Third, eNOS expression is downregulated in the monocrotaline model whereas it is upregulated in the hypoxia-induced model. Expression of eNOS mRNA in the lungs of monocrotaline-induced PH rats is reduced.[34,35] In the monocrotaline model cilostazol increased the eNOS mRNA level toward normal and attenuated PH.[34] ATRA might also upregulate eNOS from a low level toward normal in the monocrotaline model. Further studies are necessary to clarify this point.

Earlier studies and the present results showed that eNOS mRNA is increased in the lungs of chronic hypoxia-induced PH rats.[36,37] Increased plasma nitrate concentration indicates an increase in NO production on chronic hypoxic exposure. Although we could not identify the eNOS-upregulated cell type on western blot analysis because we used whole lung tissue, it might be endothelial cells of peripheral pulmonary vessels because eNOS-specific immunoreactivity was increased at this level in lung from PH rats. Because ATRA increases NOS expression in cultured endothelium,[15,16] we expected to observe the upregulation of eNOS due to ATRA in hypoxic lung tissue. But ATRA might have no effect on eNOS synthesis, at least in chronic hypoxic whole lung tissue, because there was no difference in plasma nitrate concentration nor in eNOS protein levels in the lung tissue among PH rats with and without ATRA. This suggests that combined chronic hypoxia and ATRA treatment did not enhance the eNOS expression more than chronic hypoxia-induced eNOS upregulation.

In summary, the administration of ATRA (30 mg·kg^{-1}·day^{-1}) did not prevent the development of PH in chronic hypoxia-induced PH, although a slight reduction of vascular remodeling was achieved. Combined with the results of earlier studies,[10,11,23] the therapeutic role of ATRA might depend on the etiology in preventing the development of PH.

References

1. Farber HW, Loscalzo J. Pulmonary arterial hypertension. *N Engl J Med* 2004; **351:** 1655–1665.
2. Mitani Y, Maruyama K, Sakurai M. Prolonged administration of L-arginine ameliorates chronic pulmonary hypertension and pulmonary vascular remodeling in rats. *Circulation* 1997; **96:** 689–697.
3. Roberts JD, Roberts CT, Jones RC, Zapol WM, Bloch KD. Continuous nitric oxide inhalation reduces pulmonary arterial structural changes, right ventricular hypertrophy, and growth retardation in the hypoxic newborn rat. *Circ Res* 1995; **76:** 215–222.
4. Maruyama J, Maruyama K, Mitani Y, Kitabatake M, Yamauchi T, Miyasaka K. Continuous low-dose NO inhalation does not prevent monocrotaline-induced pulmonary hypertension in rats. *Am J Physiol* 1997; **272:** H517–H524.
5. Ilkiw R, Todorovich-Hunter L, Maruyama K, Shin J, Rabinovitch M. SC-39026, a serine elastase inhibitor, prevents muscularization of peripheral arteries, suggesting a mechanism of monocrotaline-induced pulmonary hypertension in rats. *Circ Res* 1989; **64:** 814–825.
6. Maruyama K, Ye C, Woo M, Venkatacharya H, Lines LD, Silver MM, et al. Chronic hypoxic pulmonary hypertension in rats and increased elastolytic activity. *Am J Physiol* 1991; **261:** H1716–H1726.
7. Jiang BH, Maruyama J, Yokochi A, Amano H, Mitani Y, Maruyama

K. Correlation of inhaled nitric-oxide induced reduction of pulmonary artery pressure and vascular changes. *Eur Respir J* 2002; **20:** 52–58.

8. Jiang BH, Maruyama J, Yokochi A, Iwasaki M, Amano H, Mitani Y, et al. Prolonged nitric oxide inhalation fails to regress hypoxic vascular remodeling in rat lung. *Chest* 2004; **125:** 2247–2252.

9. Preston IR, Tang G, Tilan JU, Hill NS, Suzuki YJ. Retinoid and pulmonary hypertension. *Circulation* 2005; **111:** 782–790.

10. Qin Y, Zhou A, Ben X, Shen J, Liang Y, Li F. All-trans retinoic acid in pulmonary vascular structural remodeling in rats with pulmonary hypertension induced by monocrotaline. *Chin Med J* 2001; **114:** 462–465.

11. Baybutt RC, Herndon BL, Umbehr J, Mein J, Xue Y, Reppert S, et al. Effects on cytokines and histology by treatment with the ACE inhibitor captopril and the antioxidant retinoic acid in the monocrotaline model of experimentally induced lung fibrosis. *Curr Pharm Des* 2007; **13:** 1327–1333.

12. Hayashi A, Suzuki T, Tajima S. Modulation of elastin expression and cell proliferation by retinoids in cultured vascular smooth muscle cells. *J Biochem* 2004; **117:** 132–136.

13. Miano JM, Topouzis S, Majesky MW, Olson EN. Retinoid receptor expression and all-trans retinoic acid-mediated growth inhibition in vascular smooth muscle cells. *Circulation* 1996; **93:** 1886–1895.

14. Miano JM, Kelly LA, Artacho CA, Nuckolls TA, Piantedosi R, Blaner WS. All-trans-retinoic acid reduces neointimal formation and promotes favorable geometric remodeling of the rat carotid artery after balloon withdrawal injury. *Circulation* 1998; **98:** 1219–1227.

15. Achan V, Tran CT, Arrigoni F, Whitley GS, Leiper JM, Vallance P. All-trans-retinoic acid increases nitric oxide synthesis by endothelial cells: A role for the induction of dimethylarginine dimethylaminohydrolase. *Circ Res* 2002; **90:** 764–769.

16. Uruno A, Sugawara A, Kanatsuka H, Kagechika H, Saito A, Sato K, et al. Upregulation of nitric oxide production in vascular endothelial cells by all-trans retinoic acid through the phosphoinositide 3-kinase/akt pathway. *Circulation* 2005; **112:** 727–736.

17. Tatematsu S, Wakino S, Kanda T, Homma K, Yoshioka K, Hasegawa K, et al. Role of nitric oxide-producing and -degrading pathways in coronary endothelial dysfunction in chronic kidney disease. *J Am Soc Nephrol* 2007; **18:** 741–749.

18. Kurtz PJ, Emmerling DC, Donofrio DJ. Subchronic toxicity of all-trans-retinoic acid and retinylidene dimedone in Sprague-Dawley rats. *Toxicology* 1984; **30:** 115–124.

19. McPhillips DM, Lindamood C 3rd, Heath JE, Hill DL. The disposition and toxicology of retinyl methyl ester in rats dosed orally. *Drug Metab Dispos* 1988; **16:** 683–689.

20. Sani BP, Meeks RG. Subacute toxicity of all-trans- and 13-cis-isomers of N-ethyl retinamide, N-2-hydroxyethyl retinamide, and N-4-hydroxyphenyl retinamide. *Toxicol Appl Pharmacol* 1983; **70:** 228–235.

21. Festing MF, Hawkey CM, Hart MG, Turton JA, Gwynne J, Hicks RM. Principal components analysis of haematological data from F344 rats with bladder cancer fed N-(ethyl)-all-trans-retinamide. *Food Chem Toxicol* 1984; **22:** 559–572.

22. Thomas BJ, Wanstall JC. Alterations in pulmonary vascular function in rats exposed to intermittent hypoxia. *Eur J Pharmacol* 2003; **477:** 153–161.

23. Swamidas GP, Basaraba RJ, Baybutt RC. Dietary retinol inhibits inflammatory responses of rats treated with monocrotaline. *J Nutr* 1999; **129:** 1285–1290.

24. Jiang BH, Maruyama J, Yokochi A, Mitani Y, Maruyama K. A novel inhibitor of inducible nitric oxide synthase, ONO-1714, does not ameliorate hypoxia-induced pulmonary hypertension. *Lung* 2007; **185:** 303–308.

25. Rabinovitch M, Gamble W, Nadas AS, Miettinen OS, Reid L. Rat pulmonary circulation after chronic hypoxia: Hemodynamic and structural features. *Am J Physiol* 1979; **236:** H818–H827.

26. Rosenberg H, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. *Am J Physiol* 1988; **255:** H1484–H1491.

27. Nagaoka T, Fagan KA, Gebb SA, Morris KG, Suzuki T, Shimokawa H, et al. Inhaled Rho kinase inhibitors are potent and selective vasodilators in rat pulmonary hypertension. *Am J Respir Crit Care Med* 2005; **171:** 494–499.

28. Jiang BH, Tawara S, Abe K, Takaki A, Fukumoto Y, Shimokawa H. Acute vasodilator effect of fasudil, a Rho-kinase inhibitor, in monocrotaline-induced pulmonary hypertension in rats. *J Cardiovasc Pharmacol* 2007; **49:** 85–89.

29. Watanabe H. Rho-kinase activation in patients with pulmonary hypertension. *Circ J* 2009; **73:** 1597–1598.

30. Do.e Z, Fukumoto Y, Takaki A, Tawara S, Ohashi J, Nakano M, et al. Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* 2009; **73:** 1731–1739.

31. Nagaoka T, Morio Y, Casanova N, Bauer N, Gebb S, McMurtry I, et al. Rho/Rho kinase signaling mediates increased basal pulmonary vascular tone in chronically hypoxic rats. *Am J Physiol Lung Cell Mol Physiol* 2003; **287:** L665–L672.

32. Hyvelin JM, Howell K, Nichol A, Costello CM, Preston RJ, McLoughlin P. Inhibition of Rho-kinase attenuates hypoxia-induced angiogenesis in the pulmonary circulation. *Circ Res* 2005; **97:** 185–191.

33. Crossno JT, Garat CV, Reusch JE, Morris KG, Dempsey EC, McMurtry IF, et al. Rosiglitazone attenuates hypoxia-induced pulmonary remodeling. *Am J Physiol Lung Cell Mol Physiol* 2007; **292:** L885–L897.

34. Sun CK, Yuen CM, Kao YH, Chang LT, Chua S, Sheu JJ, et al. Propylthiouracil attenuates monocrotaline-induced pulmonary arterial hypertension in rats. *Circ J* 2009; **73:** 1722–1730.

35. Chang LT, Sun CK, Sheu JJ, Chiang CH, Youssef AA, Lee FY, et al. Cilostazol therapy attenuates monocrotaline-induced pulmonary arterial hypertension in rat model. *Circ J* 2008; **72:** 825–831.

36. Le Cras TD, Xue C, Renqasamy A, Johns RA. Chronic hypoxia upregulates endothelial and inducible NO synthase gene and protein expression in rat lung. *Am J Physiol* 1996; **270:** L164–L170.

37. Tyler RC, Muramatsu M, Abman SH, Stelzner TJ, Rodman DM, Bloch KD, et al. Variable expression of endothelial NO synthase in three forms of rat pulmonary hypertension. *Am J Physiol* 1999; **276:** L297–L303.