Pulmonary hypertension (PH) is a disease associated with a poor prognosis; it is resistant to drug treatment and is characterized by the progressive elevation of pulmonary artery pressure and pulmonary vascular resistance, ultimately producing right ventricular (RV) failure and leading to death (1). Previously, a variety of drugs have been tried for treating patients with PH, such as anticoagulant and vasodilator agents (2,3). Anticoagulants are believed to reduce in situ thrombosis in the pulmonary circulation, and thus the progression of this disease may be slowed (2). As vasodilator agents, calcium-channel blockers have been used to reduce vasoconstriction of the pulmonary vasculature (3). Furthermore, other treatments, such as inotropic agents, diuretics and oxygen supplementation, have been used for patients with PH. All of these conventional therapies are partially effective in some patients; however, none of them has resulted in improved survival (4).

Prostacyclin (PGI2) is a potent, short-acting vasodilator and inhibitor of platelet aggregation that is endogenously produced by the vascular endothelium (5). Vascular tone is maintained by the balance of vasodilative and vasoconstrictive prostanooids released by platelets and, to a considerable extent, the vascular endothelium (6). An imbalance in the production of PGI2, a vasodilator, and thromboxane A2 (TXA2), a vasoconstrictor, in the pulmonary circulation exists in patients with PH, and this seems to cause the progression of PH (6,7). It has been reported that intravenous (IV) PGI2 infusion significantly reduces pulmonary arterial pressure and pulmonary vascular resistance in patients with PH (5,8). Recently, continuous IV infusion of PGI2 was reported to improve exercise capacity (4,9) and long-term survival in patients with PH (4,10), in addition to lowering pulmonary vascular resistance (11). However, it must be given continuously through a central IV catheter infusion system, which may be associated with serious
We hypothesized that the combination of an oral ET$_A$ receptor antagonist and an oral PGI$_2$ analogue would be more effective than the single use of each drug alone for ameliorating PH. Therefore, we investigated the effectiveness of these two drugs in reducing the progression of PH in an animal model, alone and in combination. A single subcutaneous injection of MCT, a pyrrolizidine alkaloid, causes pulmonary vascular endothelial cell damage and medial wall thickening of muscular pulmonary arteries, which lead to PH (22,26–28). Therefore, we used MCT-treated rats as a PH model in this study.

**METHODS**

**Study protocols.** First, four-week-old male Wistar rats were given a single subcutaneous injection of 60 mg/kg MCT (Wako Pure Chemical, Osaka, Japan) (PH rats) or saline (normal rats), according to our previously described report (22,26,27). The ET$_A$ receptor antagonist TA-0201 (29,30) and/or the PGI$_2$ analogue BPS (14), or vehicle was administered orally once per day from the day before MCT injection to 19 days after. Rats were evaluated 1, 7, 14 and 19 days after injection by two-dimensional echocardiography. Nineteen days after the start of treatment, hemodynamics were evaluated; the heart was excised and divided into the RV, interventricular septum and left ventricle (LV). The lungs were also excised and immersed in 10% buffered formalin for histologic evaluation.

Second, to determine the inhibitory effect of these drugs, which were started after the onset of PH, on disease progression, we administered the drugs from 10 days (after the onset of PH [22]) to 19 days after MCT injection.

Third, to determine whether the use of a higher dose of each drug can reach the ameliorating effect on PH progression obtained by the combination treatment of both drugs, the rats were treated with a higher dose of each drug from 1 day before to 19 days after MCT injection.

The study was approved by the Laboratory Animal Resource Center of the University of Tsukuba and conformed to the "Position of the American Heart Association on Research Animal Use," adopted by the American Heart Association on November 11, 1984.

**Study groups.** First, the rats were classified into the following five groups: 1) normal rats administered vehicle (Control group, n = 12); 2) PH rats administered vehicle (PH group, n = 17); 3) PH rats administered the oral ET$_A$ receptor antagonist TA-0201 (synthesized by Tanabe Seiyaku Co. Ltd., Saitama, Japan) at 0.5 mg/kg/day (PH + TA group, n = 18); 4) PH rats administered the oral PGI$_2$ analogue BPS (donated by Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan) at 100 mg/kg/day (PH + BPS group, n = 13); and 5) PH rats administered TA-0201 (0.5 mg/kg/day) and BPS (100 mg/kg/day) (PH + TA + BPS group, n = 18). The drugs were started the day before MCT injection.

Second, the effect of these drugs starting after the onset of complications, including recurrent IV route infections, blood clotting and severe systemic hypotension (1,4).

Beraprost sodium (BPS) is a chemically stable oral PGI$_2$ analogue whose pharmacologic profile is similar to that of PGI$_2$ (12–14). It was reported that BPS has a protective effect on the development of PH in animal models (12), and that BPS is effective in patients with primary and secondary PH (13,15). However, the effectiveness of BPS is limited and is not sufficient to treat all patients with PH.

Endothelin (ET)-1, a potent vasoconstrictor peptide derived from endothelial cells (16,17), induces the growth of vascular smooth muscle cells (17,18) and myocardial cell hypertrophy (17,18). The plasma ET-1 level is reported to be increased in patients with PH, and ET-1 is thought to play an important role in the progression of PH (19). We have reported that the expression of ET-1 in the lungs of rats with PH due to congestive heart failure was markedly increased (20) and that the high plasma ET-1 concentration in patients with PH due to congenital heart disease was normalized by successful surgical repair, accompanied by a marked improvement of pulmonary hemodynamics (21). These observations suggest that endogenous ET-1 may contribute to the increase in pulmonary vascular tone in patients with PH. Furthermore, we have shown that an ET$_A$ receptor antagonist inhibited the progression of PH and ameliorated the vascular thickening, RV hypertrophy and poor survival in rats with PH induced by monocrotaline (MCT) (22,23). We have also reported that an ET$_A$ receptor antagonist improved PH associated with congestive heart failure (20). It was reported that acute ET receptor blockade caused selective pulmonary vasodilation in human patients with PH due to chronic heart failure (24). Recently, in patients with PH (primary or associated with scleroderma), it was reported that long-term treatment with an ET receptor antagonist increased exercise capacity and improved hemodynamics (25). These findings suggest that an ET receptor antagonist is alternative treatment for PH, as well as oral PGI$_2$ analogues.
PH (10 days after MCT injection) was also investigated in the same groups: 1) Control group, n = 6; 2) PH group, n = 7; 3) PH + TA group; n = 7; 4) PH + BPS group, n = 7; and 5) PH + TA + BPS group, n = 8.

Third, experiments using higher doses of these drugs were also performed: 1) Control group, n = 6; 2) PH group, n = 8; 3) PH + TA group (TA-0201 at 1.0 mg/kg/day), n = 9; 4) PH + BPS group (BPS at 200 μg/kg/day), n = 8; and 5) PH + TA + BPS group (TA-0201 at 1.0 mg/kg/day; BPS at 200 μg/kg/day), n = 8. The drugs were started the day before MCT injection.

Two-dimensional echocardiography. The rats were laid on their back under anesthesia with diethyl ether. Two-dimensional echocardiography was performed with an echocardiographic system (Model SSD-900, Aloka, Tokyo, Japan) and a 7.5-MHz probe (UST-987-7.5, Aloka). In the parasternal echocardiographic window, a two-dimensional short-axis view of the LV was obtained at the level of the papillary muscle. To estimate the increase in RV systolic pressure, we calculated the ratio of the minor axis to the major axis of the LV in the end-systolic phase (31). Measurements were performed by a single observer.

Hemodynamic measurements. Hemodynamic parameters were measured according to our previous reports, with minor modifications (20,22,27,30). The rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). Arterial blood pressure and heart rate were monitored with a polyethylene catheter inserted into the right carotid artery, and another polyethylene catheter was inserted into the right jugular vein and advanced into the RV for measurement of RV pressure (AP-601G amplifier and WT-687G thermal pen recorder, Nihon Koden, Tokyo, Japan).

Reverse transcription polymerase chain reaction (RT-PCR). Total ribonucleic acid (RNA) from the RV was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction with ISOGEN (Nippon Gene Ltd., Tokyo, Japan), and the messenger RNA (mRNA) levels were analyzed by RT-PCR, according to our previous methods (27,30,32). In the myocardium, there are two subunits of myosin heavy chain (MHC): alpha-MHC and beta-MHC. A distinction between alpha-MHC and beta-MHC was made by employing our previous method (32) using a PCR thermal cycler (TP-3000, TaKaRa Ltd., Otsu, Japan). The sequences of the oligonucleotides, which are identical to both subunits, were as follows: MHC (sense): 5’GCAGACCATCAAGGACCT3’; and MHC (antisense): 5’GTTCGCCCTGTTCCTCCGCCC’. The PCR reaction mixture was digested with Mac1 (New England Biolabs, Inc., Beverly, Massachusetts); the PCR product of alpha-MHC was not digested, whereas that of beta-MHC was digested. The amplified products on agarose gels were stained with ethidium bromide, visualized by an ultraviolet transilluminator and photographed. The photograph was scanned by a scanner (CanoScan 600, Canon Ltd., Tokyo, Japan), and quantification was performed using MacBAS software (FUJI FILM Ltd., Tokyo, Japan) (27,30,32).

Histologic examination of the lungs. Paraffin sections of 4 μm thickness from each left lung stained with azan were examined under light microscopy. Pulmonary arteries with an external diameter of about 50 μm were scanned, and medial wall thickness was measured on a personal computer with MacScope software (Mitani Ltd., Fukui, Japan), according to our previous methods (22). The ratio of medial wall thickness to external diameter of each artery was calculated and evaluated in each group.

Statistical analysis. All data were expressed as the mean value ± SE. All statistical comparisons were performed with a statistical package for Macintosh personal computer (STAT VIEW, version 4.5, Abacus Concepts Inc., Berkeley, California). The significance of differences was analyzed by using Kruskal-Wallis one-way analysis of variance, followed by the Fisher test of protected least significant differences for multiple comparisons. The results were considered statistically significant at p < 0.05.

RESULTS

Time course of PH evaluated by two-dimensional echocardiography. Changes in the ratio of the minor axis to the major axis of the LV in the end-systolic phase in PH rats treated with the moderate doses of drugs are shown in Figure 1. The ratio in the Control (healthy) group did not change by days 7, 14 and 19 from baseline (day 1) (Fig. 1). The ratio in the PH group was markedly decreased by day 14, and the ratio had further decreased by day 19 (Fig. 1). By day 19, the decrease in the ratio in the PH + TA and PH + BPS groups was inhibited to a similar extent as that in the PH group (Fig. 1). The decrease in the ratio in the PH + TA + BPS group was inhibited to the greatest degree among the three PH groups with treatments (Fig. 1).

Hemodynamic measurements. Right ventricular systolic pressure and the ratio of RV systolic pressure to systemic systolic blood pressure (Pp/Ps), an index of PH, were much greater in the PH group than in the Control group (Fig. 2). The indexes were comparably lower in the PH + TA and PH + BPS groups than in the PH group and were lowest in the PH + TA + BPS group among the three PH groups with treatments (Fig. 2). In rats treated with higher doses of drugs, the percent change of increase in RV systolic pressure was following: PH + TA, 60%; PH + BPS, 54%; and PH + TA + BPS, 25% (these percentages represent the change of increase as it relates to 100% in the PH group and 0% in the Control group). It showed the same tendency and similar extent as that in rats treated with moderate doses of drugs. In rats treated with moderate doses of drugs starting after the onset of PH, the percent change of increase in RV systolic pressure was following: PH + TA, 65%; PH + BPS, 83%; and PH + TA + BPS, 53%. The suppressive degree of increase in RV systolic pressure in the PH + TA + BPS group starting after the onset of PH was...
The suppressive degree of increase in the RV/BW ratio was following: PH TA, 94%; PH BPS, 35% (these percentages represent the change of increase as it relates to 100% the value in the PH group and 0% in the Control group). It showed the same tendency and similar extent as that in rats treated with moderate doses of drugs. In rats treated with moderate doses of drugs starting after the onset of PH, the degree of increase in the RV/BW ratio was following: PH TA, 94%; PH BPS, 62%; and PH BPS, 74%. The suppressive degree of increase in the RV/BW ratio in the PH TA and PH BPS group starting after onset of PH was significant but small compared with that starting before the onset of PH.

Weight measurements. The ratios of RV wet weight to body weight (BW) and RV wet weight to LV wet weight were markedly higher in the PH group than in the Control group (Fig. 3). The ratios were comparably lower in the PH TA and PH BPS groups than in the PH group and were lowest in the PH TA and PH BPS group among the three PH groups with treatment (Fig. 3). In rats treated with higher doses of drugs, the degree of increase in the RV/BW ratio was following: PH TA, 63%; PH BPS, 62%; and PH TA BPS, 35% (these percentages represent the change of increase as it relates to 100% the value in the PH group and 0% in the Control group). It showed the same tendency and similar extent as that in rats treated with moderate doses of drugs. In rats treated with moderate doses of drugs starting after the onset of PH, the degree of increase in the RV/BW ratio was following: PH TA, 94%; PH BPS, 110; and PH TA BPS, 74%. The suppressive degree of increase in the RV/BW ratio in the PH TA and PH BPS group starting after onset of PH was significant but small compared with that starting before the onset of PH.

Expression of alpha- and beta-MHC mRNA in the RV. We used the ratio of the expression of beta-MHC mRNA to alpha-MHC mRNA as a molecular marker for ventricular hypertrophy. In the first series of experiments, the ratio of the expression of beta-MHC mRNA to alpha-MHC mRNA in the RV was markedly higher in the PH group (Fig. 4). The increase was comparably depressed in the PH TA and PH BPS groups compared with the PH group (Fig. 4). Furthermore, the ratio was almost normalized in the PH TA and BPS group compared with the Control group (Fig. 4).

Histologic examination of the lungs. Medial wall thickness was about 1.4-fold greater in the PH group than in the Control group (Figs. 5 and 6). The ratio tended to be lower in the PH TA and PH BPS groups than in the PH group (vehicle treatment); however, it showed no significant difference statistically (Fig. 6). In contrast, the ratio was lowest in the PH TA and BPS group among the three PH groups, and statistical significance was observed between the PH group and the PH TA and BPS group (Figs. 5 and 6).

DISCUSSION
Superiority of combination treatment over single use of each drug. The present study revealed that, in PH rats, the combination of an ET A receptor antagonist and a PGI 2 analogue is more effective than the single use of each drug alone in inhibiting the progression of PH. This conclusion is derived from the following results: 1) the combination of an ET A receptor antagonist and a PGI 2 analogue inhibited the increase in RV systolic pressure and Pp/Ps to a greater degree than was obtained with either drug alone in PH rats, and these data were also supported by the results of echocardiography; 2) the combination of these drugs inhibited medial wall thickening of the pulmonary artery to a greater degree than was obtained with either drug alone; and 3) the combination of these drugs inhibited RV hypertrophy to a greater degree than was obtained with either drug alone, which was in accordance with the increased expression of beta-MHC mRNA. The present study also showed that combined treatment ameliorated PH, even if it started after the onset of PH. Furthermore, higher doses of these drugs (see Results) prevented PH progression to the same extent as moderate doses (Figs. 1 to 6). The use of a higher dose of either drug could not achieve the ameliorating effect of the combination treatment. Thus, the combination treatment is superior to the single use of each drug alone in ameliorating PH.

Mechanisms for favorable effects of combination treatment on pulmonary circulation. Beraprost sodium supplies PGI 2 to the pulmonary circulation, whereas TA 0201 blocks the binding of ET 1 to ET A receptors. The signal transduction system differs between PGI 2 and ET 1: PGI 2 activates adenylate cyclase and increases cyclic adenosine monophosphate, which decreases intracellular Ca 2+ (33), whereas ET 1 activates phospholipase C and diacylglycerol, followed by an increase in inositol triphosphate and activation of protein kinase C, which increases intracellular Ca 2+ (17). Thus, the supplementation of PGI 2 by BPS and blockade of ET 1 binding by TA 0201 may decrease
intracellular Ca\(^{2+}\) and dilate the pulmonary arteries through the additional mechanism of each compound. Furthermore, there is a possibility of an interaction between PGI\(_2\) and ET-1 in the induction of gene expression; for example, PGI\(_2\) is reported to inhibit expression of the ET-1 gene in endothelial cells (17,34) and ET-1–induced deoxyribonucleic acid synthesis in vascular smooth muscle cells (35). Therefore, the combined use of two compounds may inhibit the development of PH additionally and synergistically, partly through ablating the pharmacologic action of ET-1.

**Pathophysiologic involvement of the PGI\(_2\) and ET-1 pathways in PH.** One of the assumed mechanisms of the development of PH is impairment of vascular endothelial cell function of the pulmonary vasculature (6). Pulmonary vascular endothelial dysfunction leads to an imbalance of the production of PGI\(_2\) and TXA\(_2\) (decrease in PGI\(_2\) and increase in TXA\(_2\)), and this discrepancy is considered to cause vascular spasm of pulmonary capillary vessels and microthrombus formation (6,7,36). The administration of BPS supplies PGI\(_2\) to the pulmonary circulation in PH rats; therefore, vasospasm and generation of microthrombus may be inhibited. Thus, PH is ameliorated by the administration of BPS.

Activation of the ET-1 pathway in the pulmonary circulation is assumed to be involved in the progression of PH (19,22). We previously reported that the increase in pulmonary arterial pressure is partly attributable to the potent vasoconstrictive action of ET-1 (26). In addition, ET-1 has a potent proliferative effect on vascular smooth muscle cells (17,18), suggesting that the increase in pulmonary vascular resistance and pulmonary arterial pressure may be partly due to vascular smooth muscle cell proliferation and narrowing.
of the lumen. Therefore, administration of TA-0201 suppresses the progression of PH by inhibiting the proliferation of pulmonary vascular smooth muscle cells, as well as by inhibiting vasoconstriction of the pulmonary vasculature.

Mechanisms for favorable effects of combination treatment on RV hypertrophy. One of the mechanisms of inhibition of RV hypertrophy is considered to be the reduction in pulmonary arterial pressure and pulmonary vascular resistance by these compounds. Endothelin-1 is produced by cardiac myocytes and has a potent cardiac hypertrophic effect both in vitro and in vivo (6,17,18). We have also reported that pressure overload increases the production of ET-1 in the heart and that the expression of ET-1 mRNA is elevated in the hypertrophied RV of PH rats (22,27). Thus, another mechanism for the inhibition of RV hypertrophy is suspected to be partly attributable to TA-0201’s interference with the direct action of ET-1 on cardiac hypertrophy. Furthermore, as PGI2 suppresses the induction of the ET-1 gene (17,34), the administration of both compounds may inhibit RV hypertrophy additionally and synergistically. Therefore, the administration of both compounds is considered to be a good combination for the treatment of RV hypertrophy and PH, because excessive hypertrophy of the RV is regarded as a risk factor for right heart failure, which is associated with high morbidity and mortality (1).

Clinical implications. The present study showed that the combination of an oral ET_A receptor antagonist and an oral PGI2 analogue was superior to the single use of each drug alone in inhibiting the progression of PH in rats. As mentioned earlier, IV infusion of PGI2 is an effective therapy for patients with PH, however, it must be given continuously through a central IV line, and therefore several complications may develop (1,4). Thus, the use of orally available drugs is expected to become the norm, and the
combined use of an orally available ET\textsubscript{A} receptor antagonist and an orally available PGI\textsubscript{2} analogue is considered to become an important strategy, rather than continuous IV infusion of PGI\textsubscript{2} alone. Furthermore, the present study also showed that combined therapy ameliorates PH, even if it starts after the onset of PH, and this condition is commonly found in the clinical setting.

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