METHODS

Study Protocols

The study protocols are shown in Fig. 1. Four-week-old male Wistar rats were used. 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 paper (25, 30-32). The ET-A receptor antagonist TA-0201 (35, 36) and/or the PGI_2 analogue beraprost sodium (BPS) (16) or vehicle were administered orally once per day. Oral administration was initiated on the day before MCT injection, and the administration period was for 19 days. Rats were evaluated 1, 7, 14, and 19 days after injection by two dimensional echocardiography. Nineteen days after beginning the treatment, rats were anesthetized and hemodynamics were evaluated. After hemodynamic measurement, the heart was excised and divided into the right ventricle (RV), interventricular septum (IVS), and left ventricle (LV). Each ventricle was weighed and rapidly frozen in liquid nitrogen. The tissue samples were stored at -80°C until determination of the mRNA expression of myosin heavy chain (MHC), a molecular marker for cardiac hypertrophy, by reverse transcription (RT)- polymerase chain reaction (PCR). To evaluate the histological changes in the lungs, the lungs were also excised, weighed, and immersed in 10% buffered formalin.

Study Groups

The rats were divided 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 oral ET-A receptor antagonist (TA-0201 : gift from Tanabe Seiyaku Co. Ltd., Saitama, Japan); 0.5 mg/kg/day (PH+TA group, n=18), [4] PH rats administered oral PGI_2 analogue (beraprost sodium: BPS : gift from Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan); 100 μg/kg/day (PH+BPS group, n=13), and [5] PH rats administered TA-0201 (0.5 mg/kg/day) and BPS (100 μg/kg/day) (PH+TA+BPS group, n=18). The dose of each drug was determined from preliminary experiments; in brief, the dose of each drug was above the showing sufficient potency to attenuate PH.

Two Dimensional Echocardiography

On the day of the evaluation, the rat was laid on its back under anesthesia with inhalation of diethyl ether. The chest was shaved, and 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 view of echocardiography, a two-dimensional short-axis view of LV was obtained at the level of the papillary muscle. With an elevated RV pressure, the IVS shifts toward the LV and appears flattened in the end-systolic phase. To evaluate the increase of RV systolic pressure, we calculated the ratio of the short diameter to the long diameter of LV in the end-systolic phase according to references 37 and 38. Measurements were performed by a single observer.

Hemodynamic Measurement

The hemodynamic parameters were measured according to our previous papers with minor modifications (23, 25, 31, 32, 36, 39, 40). In brief, on the day of the experiment, the rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A polyethylene catheter was inserted into the right carotid artery. After arterial blood pressure and heart rate were monitored, another polyethylene catheter was inserted into the right jugular vein and advanced into the RV for the evaluation of RV pressure. These hemodynamic measurements were recorded with a polygraph system (AP-601G amplifier and WT-687G thermal pen recorder, Nihon Koden, Tokyo, Japan).

Reverse Transcription - Polymerase Chain Reaction

Total tissue RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction with ISOGEN (Nippon Gene Ltd., Tokyo, Japan) according to the methods described in our previous papers (31, 32, 36, 41, 42). The tissue was homogenized in ISOGEN (100 mg tissue per 1 ml ISOGEN) with a Polytron tissue homogenizer (PT10SK/35, Kinematica Inc., Lucerne, Switzerland). Homogenization was followed by chloroform extraction, isopropanol precipitation, and 80% (v/v) ethanol washing of precipitated RNA. The obtained RNA was resolved in diethylpyrocarbonate-treated water, treated with DNase I (TaKaRa Ltd., Otsu, Japan), and extracted

again using ISOGEN to eliminate the genomic DNA. The RNA concentration was determined spectrophotometrically at 260 nm. Total RNA (5 μg) was primed with 0.05 μg oligo-d(T)$_{12-18}$ and reverse transcribed using avian myeloblastosis virus reverse transcriptase using a First-strand cDNA Synthesis Kit (Life Sciences, Inc., Florida, USA) according to the methods described in our previous papers (31, 32, 36, 41, 42). The reaction was performed at 43°C for 60 minutes. The cDNA was diluted in a 1:10 ratio, and 1 μl was used for PCR.

The expression of α-MHC and β-MHC mRNA was analyzed by RT-PCR. Distinction between α-MHC and β-MHC was determined by our previous method (41, 42). The principle of which is based on the assumption that one set of oligonucleotide primers will anneal to identical sequences on the α- and β-MHC transcripts with equal efficiencies; since the lengths of elongation are identical, the amplified fragments should be representative of endogenous levels of mRNA for these two transcripts. The relative proportions of the amplified DNA fragments are determined by differential restriction endonuclease digestion with an enzyme for which a restriction site is found only in one of the fragments.

Each PCR reaction contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each gene-specific primer, and 0.025 U/μl Taq polymerase (TaKaRa Ltd.). The gene-specific primer was synthesized according to the published cDNA sequences (43). The sequences of the oligonucleotides were as follows:

MHC (sense) : 5'GCAGACCATCAAGGACCT3' ,

MHC (antisense) : 5'GTTGGCCTGTTCCTCCGCC3'.

PCR was carried out using a PCR thermal cycler (TP-3000, TaKaRa Ltd.). The cycle profile included denaturation for 15 seconds at 94°C, annealing for 15 seconds at 63°C, and extension for 45 seconds at 72°C. The reaction cycles of PCR were performed in the range that demonstrates a linear correlation between the amount of cDNA and the yield products of PCR. The specificity of the amplified sequences was confirmed by restriction enzyme analysis and DNA sequencing. Distinction between α-MHC and β-MHC was achieved by digestion of 12.5 μl of the PCR reaction mixture with 0.8 U *Mse* I (New England Biolabs, Inc., Beverly, MA. USA) in a standard

reaction buffer at 37°C for 4 hours according to our previous papers (41, 42). These yielded fragments were 310 bp for α-MHC and 257+53 bp for β-MHC.

Quantitative Analysis of PCR Products

The amplified products were electrophoresed on 2.0% agarose gels, stained with ethidium bromide, visualized by a UV transilluminator, and photographed according to the methods described in our previous papers (31, 32, 36, 41, 42). The PCR products were electrophoresed on the same gel in each comparison study. The photograph was scanned by a scanner (CanoScan 600, Canon Ltd., Tokyo Japan), and quantification was performed by a personal computer with MacBAS software (FUJI FILM Ltd., Tokyo, Japan) according to our previous papers (31, 32, 36, 41, 42).

Histology of the Lungs

Paraffin sections of 4-μm thickness from each left lower lobe were stained with azan, and examined under light microscopy. Statistical analysis of medial wall thickness was examined in the sections stained with azan. In brief, a view of pulmonary arteries with about 50 μm in external diameter was scanned under light microscopy, and medial wall thickness was evaluated by a personal computer with MacScope software (Mitani Ltd., Fukui, Japan) according to the methods described in our previous paper (25). The ratio of medial wall thickness to external diameter of each artery was calculated and evaluated in each group.

Statistical Analysis

All data were presented as mean ± S.E. values. All statistical comparisons were performed with a commercially available statistical package for Macintosh personal computer (STAT VIEW, version 4.5, Abacus Concepts Inc., Berkeley, CA. USA). The significance of differences was analyzed by Kruskal-Wallis one-way analysis of variance (ANOVA), followed by Fischer's Protected Least Significant Difference for multiple comparisons. The results were considered statistically significant at the level of $p<0.05$.