FULL PAPER *Pharmacology*

Effect of Long-term Administration of a Prostacyclin Analogue (Beraprost Sodium) on Myocardial Fibrosis in Dahl Rats

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ABSTRACT. Beraprost sodium (BPS) is an orally active prostacyclin analogue. The effects of BPS on the heart, including coronary circulation improvement, myocardial and vascular protection and anti-fibrosis effect on myocardium interstitium, have previously been demonstrated. However, the effects of BPS on hemodynamics, cardiac function and myocardial contractility in patients in the hypertrophic phase have not been clarified. Therefore, in the present study, the effects of BPS under long-term administration were investigated using the hypertension model of salt-sensitive Dahl rats. Six-week-old Dahl rats were divided into three groups, an 8% high salt diet group treated with BPS (BPS group), an untreated 8% high salt diet group (HHF group) and an untreated 0.3% low salt diet group (Control group), and observations were conducted until 17 weeks of age. In the BPS and HHF groups, the survival rates after 11 weeks of high salt diet intake were 87.5% and 47.1%, respectively ($p<0.05$). At 17 weeks of age, the atrial systolic peak velocity/early diastolic peak velocity and heart weight index of the BPS group decreased significantly compared with the HHF group ($p<0.05$). The HHF group exhibited significantly more severe myocardial fibrosis mainly in the endocardial layer of the left and right ventricles compared with the BPS and Control groups ($p<0.05$). In the present study, long-term BPS administration preserved diastolic function and prevented myocardial interstitial fibrosis in the non-compensatory phase. The results of the present study suggest that BPS is effective for treatment of hypertensive cardiac hypertrophy.

KEY WORDS: beraprost sodium, Dahl rat, echocardiography, hypertension, myocardial fibrosis.

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Prostacyclin is found in all body tissues and body fluids and is the major metabolite of arachidonic acid in the vasculature [6, 16]. It is a potent vasodilator that affects both systemic and pulmonary circulations. Prostacyclin also prevents vascular smooth muscle proliferation and inhibits platelet adhesion and aggregation [6]. These features make it a very attractive substance for treatment of various cardiovascular diseases [6, 15, 16, 20]. Beraprost sodium (BPS) is an orally active prostacyclin analogue that was discovered and developed by Toray Industries in Japan [1, 16]. In human medicine, long-term administration of BPS has been approved as a treatment for chronic arterial occlusion [16] and primary pulmonary hypertension [2, 8, 16]. In addition, oral administration of BPS can be used as a therapeutic treatment for secondary precapillary pulmonary hypertension [17], cerebral infarction [9, 13], glomerulonephritis [12, 28], diabetic nephropathy [29] and atherosclerotic vascular damage in coronary artery disease [23]. In heart failure with pressure overload, compensated concentric hypertrophy in the left ventricular progresses due to the increase in pressure after overload. As a result, then the interstitial fibrosis changes the myocardial structure [21, 26, 27]. In addition, the changes in myocardial structure inhibit myocardial diastolic and systolic function, resulting in cardiac dysfunction [26, 27]. The effects of BPS, such as coronary circulation improvement, myocardial and vascular protection [19] and its anti-fibrosis effect on myocardial interstitium [30], have been demonstrated previously. However, the effects of BPS on hemodynamics, cardiac function and myocardial contractility in patients in the hypertrophic phase have not been clarified. Therefore, in the present study, we investigated the effect of long-term BPS administration on myocardial fibrosis using a hypertension model of salt-sensitive Dahl rats.

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MATERIALS AND METHODS

Animals: Dahl rats, aged 5 weeks of age, were supplied from SEAC Yoshitomi (Fukuoka, Japan). The rats were housed two or three per cage with free access to water and were maintained at a temperature of 23°C. The laboratory animals were handled and cared for in accordance with the standards established by the Tokyo University of Agriculture and Technology as described in its"Guide for the care and use of laboratory animals".

Procedures: The Dahl rats were maintained with a low salt diet possessing an inclusion rate of 0.3% NaCl (Oriental Yeast Co., Ltd. Tokyo, Japan) until they were 6 weeks old. The rats were then randomly divided into the following three groups: an 8% high salt diet group treated with BPS (Toray, Japan; BPS group, n=14), an untreated 8% high salt

diet group (HHF group, n=17) and an untreated 0.3% low salt diet group (Control group, n=12). The animals were observed until 17 weeks of age.

Administration: Daily water intake was calculated for each group. BPS was dissolved in distilled water and administrated with free access to water at 300 μg/kg/day throughout the observation period.

Observations: The general physical conditions of the animals, including clinical signs and survivability, were observed daily, and body weight was measured weekly during the course of the study. Necropsy was performed on animals that died during the course of this study.

Echocardiography: Color Doppler and M-mode echocardiographic examinations were conducted at 6, 13 and 17 weeks of age using an ultrasound scanner (SSD-5000, ALOKA, Tokyo, Japan) with a 10.0 MHz transducer. The animals were sedated with xylazine hydrochloride (10 mg/kg, intraperitoneal) and ketamine hydrochloride (50 mg/kg, intraperitoneal). End-diastolic left ventricular internal dimension (LVIDd), end-diastolic interventricular septum thickness (IVSd) and end-diastolic left ventricular posterior wall dimension (LVPWd) were measured using M-mode echocardiographic images of the left ventricular short axis view in right lateral recumbency. The LV mass was then calculated using the following formula [5, 18]:

$$\text{LV mass} = 1.04 \times (\text{LVIDd}+\text{LVPWd}+\text{IVSd})^3 - \text{LVIDd}^3].$$

This parameter was indexed to body weight (LV mass index). Next, in the short axis view of the heart base, the peak pulmonary arterial velocity was measured using pulsed-wave Doppler echocardiography. The right ventricular stroke volume was calculated from the integral of the wave form and pulmonary valve orifice area. In addition, atrial systolic peak velocity/early diastolic peak velocity (A/E) and E-wave decelerating time (DT) were measured from the apex four chamber view in left lateral recumbency.

Blood pressure measurement: The rats were anesthetized with pentobarbital (25 mg/kg, intraperitoneal), and anesthesia was maintained by inhalation of 2% isoflurane. Arterial blood pressure was measured by direct arterial sphygmomanometry at 13 and 17 weeks of age. A 24-gauge vein catheter was placed in the left carotid artery, and the systolic and diastolic blood pressure were measured. Right and left ventricular pressure was measured using a 22 gauge vein catheter inserted into the apex and right ventricular outflow tract, respectively, at 17 weeks of age under thoracotomy with artificial ventilation.

Histology and morphometry: After blood pressure was measured at 17 weeks of age, potassium chloride (1 mol/*l*) was injected via the vein catheter inserted into the apex of the left ventricle, and the heart was arrested in diastole. The heart was immediately removed, weighed and fixed in 10% buffered formalin for three days. On the median level of the left ventricular papillary muscle, the ventricles (2–3 mm in depth) were dissected out horizontally towards the coronary groove, which was then dehydrated with ethanol and embedded in paraffin. Five μm thick sections were removed and stained with hematoxylin and eosin and red picrosirius-stain. To measure the area of fibrosis in the sections stained with red picrosirius, 15 fields per section were chosen and the left ventricular endocardial layer, median layer, epicardial layer and right ventricle were measured in each field. Digital photomicrographs (100 × magnification) were obtained with a digital camera (KS-630; Olympus), and the areas of fibrosis and calcification were calculated using image processing software (Mac Scope, Mitani Corporation, Japan). The area of fibrosis was expressed as percentage of the microscopic field.

Statistical Analysis: The results were expressed as means ± SEM. All data were analyzed statistically using Thompson's method. Differences among the three groups were assessed using one-way ANOVA and the Tukey-Kramer multiple comparison test. Survival curves were constructed for each group using the Kaplan-Meier Method, and comparison of survival distributions among the three groups was performed using the log-rank test. A value of $p<0.05$ was used to indicate a statistically significant difference.

RESULTS

Clinical progress and survival rate: In the BPS and HHF groups, body weight exhibited a tendency to decrease compared with the Control group during the experiment; however, no significant differences were observed between the HHF and BPS groups. The survival rates of the BPS (n=14) and HHF groups (n=17) at 17 weeks of age were 87.5% and 47.1%, respectively ($p<0.05$, Fig. 1). The rats that died during the study showed inanition, dyspnea and tachypnea a few days prior to their death. Subsequent lesions were found in necropsy, including subcutaneous edema, reddish brown serous pleural effusion and pulmonary edema, that were suggestive of left and right heart failure. The Control group, on the other hand, were in good condition and all sur-

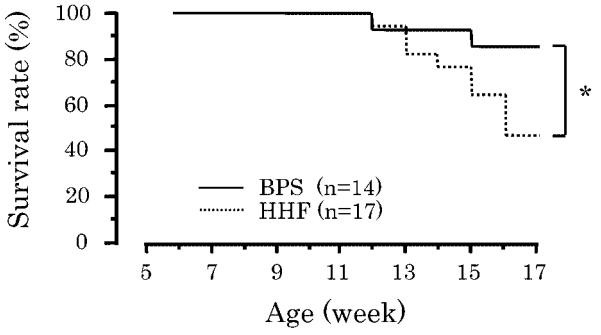

Fig. 1. Survival curve of the Dahl rats (Kaplan-Meier method). At 6 weeks of age, the animals were divided into three groups, and survival rate was calculated at 17 weeks of age using the log-rank test. BPS: 8% high salt diet and treated with beraprost sodium. HHF: untreated 8% high salt diet and untreated group. CTRL: 0.3% untreated low salt diet group. Significant difference between BPS and HHF at $p<0.05$ (*).

Table 1. Values of echoardiogrphic parameters, blood pressures and heart weights indexed to body weight

	13 weeks			17 weeks		
	Control	BPS	HHF	Control	BPS	HHF
LVPWd (mm)	1.65 ± 0.08	2.26 ± 0.09**	2.38 ± 0.09**	1.65 ± 0.07	2.26 ± 0.05**	2.31 ± 0.09**
IVSd (mm)	1.57 ± 0.07	2.16 ± 0.08**	2.11 ± 0.08**	1.76 ± 0.03	2.16 ± 0.05**	2.10 ± 0.13**
LV mass index (cm^3/g)	0.15 ± 0.01	0.22 ± 0.02**	0.21 ± 0.01**	0.09 ± 0.01	0.17 ± 0.02*	0.17 ± 0.03**
A/E	0.64 ± 0.04	0.77 ± 0.04*	0.75 ± 0.05	0.66 ± 0.04	0.79 ± 0.03*†	0.95 ± 0.06**
Arterial pressure (mmHg)						
Systole	111.3 ± 7.0	140.9 ± 10.4*	136.1 ± 7.4*	130.1 ± 5.2	131.9 ± 15.1	137.2 ± 78.8
Diastole	89.7 ± 7.6	117.8 ± 9.9*	114.7 ± 8.0*	105.8 ± 4.6	106.0 ± 14.2	117.8 ± 17.6
Left ventricular systolic pressure (mmHg)	–	–	–	86.0 ± 6.2	116.2 ± 13.1*	103.6 ± 5.2
HWI	–	–	–	29.4 ± 0.6	48.0 ± 3.5*†	62.4 ± 4.5**

Values were expressed as means ± SEM. Significant difference compared to Control at p<0.05 (*) and p<0.01 (**) and compared to HHF at p<0.05 (†). LVPWd: End-diastolic left ventricular posterior wall dimension. IVSd: End-diastolic interventricular septum thickness. LV mass index: Left ventricular mass indexed to body weight. A/E: Atrial systolic velocity/early diastolic velocity into the left ventricle. HWI: Heart weight per 100 g body weight.

vived to the end of the experiment.

Echocardiography: No significant difference in left ventricular fractional shortening was observed among the three groups. In the BPS and HHF groups, LVPWd (p<0.01) and IVSd (p<0.01) increased significantly compared with the Control group (Table 1) at 13 and 17 weeks of age. However, there was no significant difference between the BPS and HHF groups. The LVIDd did not differ significantly among the three groups at 17 weeks of age (data not shown). In addition, in the BPS and HHF groups, the LV mass index increased significantly compared with the Control group at 13 and 17 weeks of age (Table 1). In the BPS and HHF groups, A/E were 0.77 ± 0.04 and 0.75 ± 0.05 by 13 weeks of age (BPS group: p<0.05 vs Control group), respectively, and their values showed a "relaxation delay pattern". In the HHF group, A/E increased significantly with the mean value reaching 0.95 ± 0.06 at 17 weeks of age (p<0.01 vs Control group; p<0.05 vs BPS group). On the other hand, in the BPS group, A/E did not progress in a manner similar to the HHF group at 17 weeks of age (Fig. 2). No significant differences in decelerating time were observed among the three groups at 13 and 17 weeks of age.

Blood pressure: In the BPS and HHF groups, the systemic diastolic and systolic blood pressures increased significantly compared with the Control group at 13 weeks of age (p<0.05, Table 1). However, there were no significant differences among the BPS, HHF and Control groups at 17 weeks old of age. Furthermore, in the BPS group, the left ventricular systolic pressure increased significantly compared with the Control group (p<0.05, Table 1). There were no significant differences between the systemic diastolic and systolic pressures and left and right ventricular systolic pressures of the BPS and HHF groups.

Histology and morphology: The heart weight per 100 g body weight (heart weight index; HWI) of the BPS group (48.0 ± 3.5) decreased significantly compared with the HHF group (62.4 ± 4.5) (p<0.05), although it increased significantly compared with the Control group (29.4 ± 0.6, p<0.05). The HHF group exhibited severe myocardial fibrosis in the endocardial (3.45 ± 0.19) and median layers of the left ventricle (3.34 ± 0.19) and in the right ventricle (3.93 ± 0.20). On the other hand, in the BPS group, inhibition of myocardial fibrosis was observed in these areas. The percentages of fibrosis in the HHF group were significantly higher compared with the value of BPS and Control groups (Figs. 3, 4).

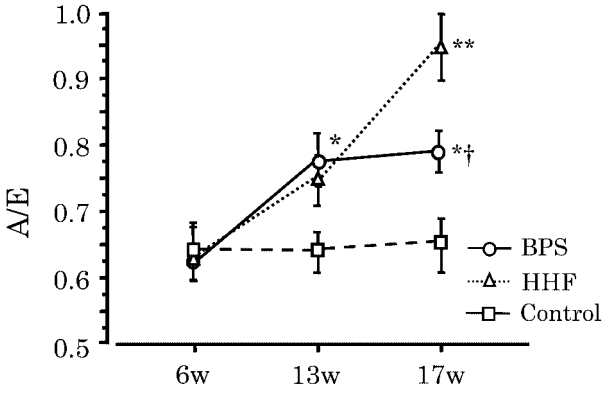

Fig. 2. Atrial systolic velocity/early diastolic velocity (A/E) into the left ventricle at 6, 13 and 17 weeks of age. Values were expressed as means ± SEM. There were significant differences among the BPS, HHF and Control groups. *: p<0.05 vs. Control. **: p< 0.01 vs. Control. †: p< 0.05 vs. HHF.

Relationship between echocardiography and myocardial fibrosis: There were significant correlations between A/E (r=0.61, p<0.01), IVSd (r=0.75, p<0.001), LVPWd (r=0.64, p<0.01), LV mass index (r=0.72, p<0.01) and cardiac muscle interstitial fibrosis. However, there were no significant

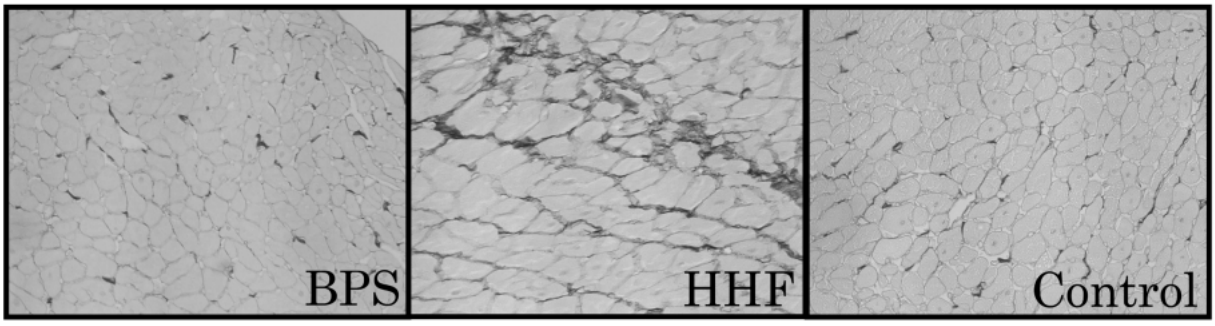

Fig. 3. Section of the left ventricular endocardial layer stained with red picrosirius (× 100).

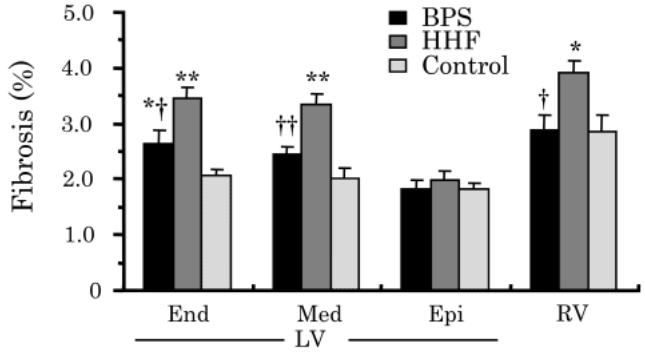

Fig. 4. Measurement of the area of fibrosis stained with red picrosirius. Values were expressed as means ± SEM. There were significant differences among the BPS, HHF and Control groups. End: left ventricular (LV) endocardial layer. Med: LV median layer. Epi: LV epicardial layer. RV: right ventricle. *: $p<0.05$ vs. Control. **: $p<0.01$ vs. Control. †: $p<0.05$ vs. HHF. ††: $p<0.01$ vs. HHF.

correlations between LVIDd and DT.

DISCUSSION

In the present study, long-term administration of BPS was shown to be effective in treating sodium chloride-sensitive hypertension and a significant improvement in survival rate was achieved in the BPS group compared with the HHF group. In order to determine the reasons for this improvement in the survival rate, two phases of cardiac hypertrophy, and systolic and diastolic functions can be considered.

The BPS-treated animals exhibited the highest value for left ventricular systolic pressure at 17 weeks of age. It has been reported that BPS has a positive inotropic effect in cardiac muscle isolated from the guinea pig [25]. Thus, BPS administration may improve cardiac muscular systolic function. On the other hand, in the BPS group, the right ventricular systolic pressure did not increase significantly compared with the Control group. It has been suggested that pulmonary vascular dilatation and increased blood flow in the pulmonary vessel bed occurs, thereby improving pulmonary circulation [22]. In the BPS and HHF groups, the LV mass index was significantly different compared with that of the Control group at 13 and 17 weeks of age, and this thought to be attributed to concentric hypertrophy. However, the diastolic functions of the BPS and HHF groups were different. Compared with the Control and BPS groups, a significant increased in A/E at 17 weeks of age suggests decreased diastolic function. In the BPS group, A/E did not increase and diastolic function was remained stable between 13 and 17 weeks of age; myocardial fibrosis was inhibited in the BPS group compared with the HHF group at 17 weeks of age. In addition, since A/E was correlated with myocardial fibrosis in terms of histology, BPS may have inhibited progression of myocardial fibrosis and maintained cardiac systolic and diastolic functions. In order to explain the mechanism for those results, the two phases of cardiac hypertrophy under the pressure overload should be considered. The first is the compensatory phase, in which myocardial hypertrophy is observed against after overload, and the second is the non-compensatory phase, in which myocardial hypertrophy occurs mainly through myocardial fibrosis [11, 31]. As BPS dose not have an antihypertensive effect, the cardiac hypertrophy observed at 13 weeks of age seems to

depend on blood pressure (the compensatory phase). On the other hand, the cardiac hypertrophy observed from 13 to 17 weeks of age appears to be the stage by the progression of fibrosis (the non-compensatory phase). Since BPS can suppress cardiac fibrosis, cardiac hypertrophy in the non-compensatory phase can be inhibited. For this reason, the inhibitory effect of BPS on diastolic dysfunction was not evident during the compensatory phase and cardiac hypertrophy progressed. However, in the non-compensatory phase, fibrosis and stiffness of the myocardial interstitium were suppressed by BPS administration and diastolic function was maintained [3, 24]. The changing point from the compensatory phase to the non- compensatory phase was determined by arterial stiffness induced by pressure overload or cardiac hypertrophy through coronary circulation failure [4, 10]. BPS effectively suppresses myocardial degeneration and fibrosis in chronic heart failure by improvement of coronary circulation and an anticytokine effect. In Dahl rats, endothelial NO production was reduced, NO degradation was accelerated [7], and kallikrein-kinin and prostacyclin activity was reduced [14] pathophysiologically. Prostacyclin analogue could efficiently improve the myocardial microenvironment under these conditions.

In the present study, the long-term BPS administration was shown to suppress myocardial interstitial fibrosis in the non-compensatory phase and prevent progression of heart failure derived from decreased diastolic function. Therefore, BPS can be effective for improving the survival rates of animals with hypertensive cardiac hypertrophy.

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