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Role of endothelin ET_B receptor in the pathogenesis of monocrotaline-induced pulmonary hypertension in rats

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Abstract

We investigated the role of endothelin ET_B receptor in the development of monocrotaline-induced pulmonary hypertension, by using the spotting-lethal (*sl*) rat, which carries a naturally occurring deletion in the endothelin ET_B receptor gene. Three weeks after injection of saline or monocrotaline (60 mg/kg, s.c.), hemodynamics, cardiac hypertrophy and endothelin-1 levels in right ventricle were determined. Monocrotaline produced a marked pulmonary hypertension associated with increases in right ventricular pressure and hypertrophy, pulmonary arterial medial thickening and the endothelin-1 levels. These monocrotaline-induced alterations tended to be enhanced in ET_B-deficient homozygous rats, compared with cases in wild-type rats. The treatment with the selective ET_A receptor antagonist ABT-627 [2*R*-(4-methoxyphenyl)-4*S*-(1,3-benzodioxol-5-yl)-1-(*N,N*-di(n-butyl)aminocarbonyl-methyl)-pyrrolidine-3*R*-carboxylic acid] for 3 weeks (10 mg/kg/day, twice daily) almost completely suppressed the monocrotaline-induced pulmonary hypertension and related organ damage both in ET_B-deficient and wild-type animals to the same levels. Thus, we suggest that the antagonism of the ET_A receptor is essential for the protection from monocrotaline-induced pulmonary hypertension, irrespective of the presence of the ET_B receptors, although a protective role of ET_B receptor-mediated action in the pathogenesis of this disease model cannot be ruled out.

Keywords: Endothlin-1; ET_A receptor; ET_B receptor; ET_B receptor-deficient rat; Pulmonary hypertension; Monocrotaline

1. Introduction

Endothelin-1 is a potent vasoconstrictor peptide isolated from the culture supernatant of porcine aoric endothelial cells. The lung is known to synthesize endothelin-1 and to possess both ET_A and ET_B receptors, both of which may be involved in physiologic and pathophysiologic actions of endothelin-1 in the lung (Michel et al., 2003). The ET_A receptors are located on smooth muscle cells, where they mediate vasoconstriction and smooth muscle proliferation. In contrast, ET_B receptors are found on both endothelial and smooth muscle cells, where they mediate vasodilation or vasoconstriction. In addition, endothelial ET_B receptors in lung are responsible for circulating endothelin-1 clearance, with close to 50% removal during the pulmonary transit in man (Dupuis et al., 1996).

There is accumulating evidence that endothelin-1 is closely related to the development of the pulmonary hypertension (Michel et al., 2003). Circulating endothelin-1 levels are increased in humans who have primary and secondary pulmonary hypertension (Stewart et al., 1991; Cody et al., 1992) and correlate well with the severity of the disease. In monocrotaline-treated rat pulmonary hypertension models, endothelin-1 concentrations were elevated in their lung perfusate compared with the case of the control animals (Frasch et al., 1999). In same animal models, cardiac endothelin-1 mRNA expression and endothelin-1 peptide

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levels in heart and plasma were known to be elevated (Miyauchi et al., 1993; Jasmin et al., 2003). The effectiveness of both selective ET_A and nonselective ET_A/ET_B receptor antagonists has been convincingly demonstrated in monocrotaline-induced (Miyauchi et al., 1993; Tilton et al., 2000; Jasmin et al., 2001; Jasmin et al., 2003) and hypoxic pulmonary hypertension (Bonvallet et al., 1994; Sato et al., 2000; Tilton et al., 2000) in rats. Recent double-blind, placebo-controlled study in patients with pulmonary hypertension confirmed the therapeutic potential of the nonselective ET_A/ET_B receptor antagonist bosentan, which improved pulmonary hemodynamics and exercise capacity (Rubin et al., 2002). Bosentan is now approved for the treatment of pulmonary hypertension in humans.

However, based on an increasing body of experimental evidence, it remains unclear which type of selective ET_A-receptor blockade or nonselective ET_A/ET_B-receptor blockade would be preferable. Moreover, although there is general agreement that ET_A receptor-mediated action plays a crucial role in the development of pulmonary hypertension, the pathophysiological role of ET_B receptors in pulmonary hypertension is not fully elucidated. Therefore, we investigate the pathophysiological role of ET_B receptors in monocrotaline-induced pulmonary hypertension, using the spotting-lethal *(sl)* rats, which carries a naturally occurring deletion in the endothelin ET_B receptor gene (Gariepy et al., 1996). Since homozygous (*sl*/*sl*) rats do not live beyond 1 month because of intestinal aganglionosis and resulting intestinal obstruction, dopamine β-hydroxylase promoter was used to direct ET_B transgene expression in *sl*/*sl* rats to support normal enteric nervous system development (Gariepy et al., 1998). These transgenic *sl*/*sl* rats live into adulthood and are healthy, expressing ET_B receptors in adrenal glands and other adrenergic neurons. They are ET_B-deficient in other tissues, but most important is the deficiency in the vascular endothelium and vascular smooth muscle (Gariepy et al., 2000). In a recent study, the lungs of these rescued ET_B-deficient homozygous (*sl*/*sl*) rats are found to lack ET_B mRNA in the pulmonary vasculature, to have minimal ET_B receptor-binding activity, and to lack endothelin-1-induced pulmonary vasodilation (Ivy et al., 2001). Moreover, we noted that an ET_B receptor-selective agonist sarafotoxin S6c-induced vasodilation was not observed in isolated perfused lung of *sl*/*sl* rats (unpublished observation). Thus, the "rescued" ET_B receptor-deficient rat is a useful tool in determining the pathophysiological roles of ET_B receptors in monocrotaline-induced pulmonary hypertension.

2. Materials and methods

2.1. Animals

The creation of DβH-ET_B transgenic rats has been described previously (Gariepy et al., 1998). Homozygous (*sl*/*sl*) rats have dark eyes and pigmented coats only in small spots on their heads. Wild-type (+/+) rats have pigmented heads, backs, and tails. To definitively differentiate these rats, polymerase chain reaction was performed on DNA isolated from tail biopsy specimens, as described (Gariepy et al., 1998).

ET_B-deficient homozygous (*sl*/*sl*) and wild-type (+/+) rats (7 weeks old), all of which were DβH-ET_B transgenic, received a subcutaneous injection of 60 mg/kg monocrotaline or saline. Monocrotaline-treated animals were gavaged twice daily with ABT-627 (a selective ET_A receptor antagonist; 10 mg/kg/day) (Jarvis et al., 2000) or vehicle (a mixture of 10% ethanol, 40% propylene glycol, and 50% distilled water), starting 24 h before the subcutaneous injection of monocrotaline and subsequently for 3 weeks.

All animals were allowed free access to standard laboratory rat chow and tap water and were housed under controlled humidity, temperature and a 12-h light/dark cycle. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Research Committee at Osaka University of Pharmaceutical Sciences.

2.2. Experimental protocol

Three weeks after the injection of monocrotaline or saline, each rat was artificially ventilated under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). A polyethylene catheter, connected to a pressure transducer was inserted into the right carotid artery to measure arterial blood pressure recorded by means of polygraph system (RM 6000, Nihon Koden, Tokyo, Japan). Another polyethylene catheter was inserted into the right jugular vein to measure right ventricular systolic pressure. The heart and lung were excised, weighed and used for morphometric analysis. A portion of right ventricle was frozen separately for determination of endothelin-1 content.

2.3. Histological studies

Excised left lungs were processed for light microscopic observation, according to standard procedures. The lungs were then preserved in phosphate-buffered 10% formalin, after which the lungs were chopped into small pieces, embedded in paraffin, cut at 3 μm and stained with Elastica-van-Gieson technique. The resistance pulmonary arteries were identified as vessels with two clearly defined elastic laminae, with layer of smooth muscle cells between two laminae. The percent wall thickness (% wall thickness) of arteries (in the size ranges of 50–100 and 100–150 μm in external diameter) was calculated by using the following formula: % wall thickness=2×wall thickness/external diameter×100 (Ono and Voelkel, 1991). The wall thickness was determined by using an image analyzer (AE-6905C, ATTO, Tokyo, Japan). For each animal, 15–20 vessels were

counted, and an average was calculated. Evaluations were made in a blind manner.

2.4. Endothelin-1 measurement

Endothelin-1 was extracted from the right ventricle, as described elsewhere (Fujita et al., 1995). Briefly, right ventricle tissue was weighed and homogenized for 60 s in 4 ml of ice-cold organic solution (chloroform/methanol, 2:1, including 1 mM *N*-ethylmaleimide). The homogenates were left overnight at 4 °C and then 0.4 ml of 0.09% trifluoroacetic acid (TFA) was added to the homogenates. Homogenates were centrifuged at 3000 rpm for 30 min and the supernatant was stored. Aliquots of the supernatant were diluted 1/10 with a 0.09% TFA solution and applied to Sep-Pak C18 cartridges. The sample was eluted with 3 ml of 63.3% acetonitrile and 0.1% TFA. Eluates were dried in a centrifugal concentrator, and the dried residue was reconstituted in assay buffer for radioimmunoassay (RIA). The clear solution was subjected to RIA. The recovery of endothelin-1 was approximately 80%. RIA for tissue endothelin-1 was done, as described elsewhere (Matsumura et al., 1990), using endothelin-1 antiserum (a generous gift from Dr. Marvin R. Brown, Department of Medicine, University of California, San Diego, CA, USA). This serum does not cross-react with big endothelin-1 (Matsumura et al., 1990).

2.5. Drugs

ABT-627 [2*R*-(4-methoxyphenyl)-4*S*-(1,3-benzodioxol-5-yl)-1-(*N,N*-di(n-butyl)aminocarbonyl-methyl)-pyrrolidine-3*R*-carboxylic acid] was provided by Abbott Laboratories (Abbott Park, IL, USA). Monocrotaline was obtained from Sigma (St. Louis, MO, USA). Other chemicals were purchased from Nacalai Tesque (Kyoto, Japan) and Wako (Osaka, Japan).

2.6. Statistical analysis

Values are mean±S.E.M. For statistical analysis, multiple-group comparisons were performed using one-way analysis of variance followed by the Student–Newman–Keuls test. Differences were considered significant at $P<0.05$.

3. Results

3.1. Body, heart, and lung weights, and systemic hemodynamics in wild-type and ET_B-deficient homozygous (sl/sl) rats

Body weight gain in monocrotaline-treated rats tended to be less than that in saline-treated control rats, in both wild-type and ET_B-deficient *sl/sl* groups, and were not affected by 3-weeks-administration of ABT-627. Systemic hemodynamics were not different between saline- and monocrotaline-treated animals, but levels of systolic blood pressure were higher in *sl/sl* rats, compared with wild-type rats. In both wild-type and ET_B-deficient *sl/sl* groups, monocrotaline injection produced significant increases in right ventricle weight and right ventricle-to-body weight ratio as well as lung weight, indicating the development of right ventricle hypertrophy and pulmonary hypertension. Monocrotaline-induced alterations were efficiently suppressed by the daily administration of ABT-627, in both groups. There is no significant changes in left ventricle plus septum weight in all experimental groups (Table 1).

Table 1
Body, heart, and lung weights, and systemic hemodynamics

	Wild-type			ET_B-deficient		
	Control (*n*=10)	MCT (*n*=10)	MCT+ABT (*n*=6)	Control (*n*=10)	MCT (*n*=10)	MCT+ABT (*n*=6)
BW (g)	288±10	248±9[a]	253±14[a]	265±13	248±8	244±13
HR (bpm)	409±6	415±9	396±18	425±7	417±8	423±5
MAP (mm Hg)	113±6	102±2	97±4	126±4	131±4[b]	124±3[c]
RV weight (g)	0.16±0.01	0.27±0.02[d]	0.16±0.01[b]	0.13±0.01	0.27±0.01[e]	0.14±0.01[f]
RV/BW (g/kg)	0.54±0.03	1.07±0.08[d]	0.63±0.03[b]	0.50±0.02	1.12±0.07[e]	0.57±0.05[f]
LV+S weight (g)	0.55±0.02	0.53±0.02	0.50±0.03	0.47±0.02	0.46±0.02	0.50±0.02
LW (g)	1.25±0.08	1.53±0.06[d]	1.27±0.07[b]	1.22±0.06	1.67±0.07[e]	1.29±0.12[f]
LW/BW (g/kg)	4.37±0.16	6.26±0.43[a]	5.02±0.19	4.63±0.19	6.88±0.42[e]	5.29±0.41[g]

Values represent the mean±S.E.M.
MCT, monocrotaline; ABT, ABT-627; BW, body weight; HR, heart rate; MAP, mean arterial blood pressure; RV, right ventricle; LV+S, left ventricle plus septum; LW, lung weight.
[a] $P<0.05$, compared with wild-type control.
[b] $P<0.01$, compared with wild-type MCT.
[c] $P<0.05$, compared with wild-type MCT+ABT.
[d] $P<0.01$, compared with wild-type control.
[e] $P<0.01$, compared with ET_B-deficient control.
[f] $P<0.01$, compared with ET_B-deficient MCT.
[g] $P<0.05$, compared with ET_B-deficient MCT.

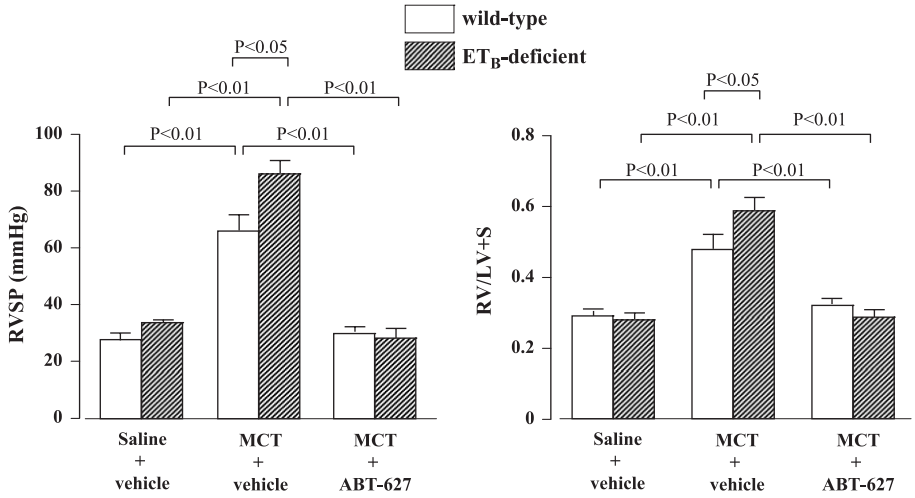

Fig. 1. The effects of ABT-627 on RV systolic pressure (RVSP) and RV-to-LV+S weight ratio (RV/LV+S) in monocrotaline (MCT)-treated wild-type and ET_B-deficient *sl*/*sl* rats 3 weeks after the MCT injection. Each column and bar represent the mean±S.E.M. (without ABT-627, n=10; with ABT-627, n=6). RV, right ventricle; LV, left ventricle.

3.2. Right ventricular systolic pressure and right ventricle-to-left ventricle plus septum weight ratio (RV/LV+S) in wild-type and ET_B-deficient homozygous (sl/sl) rats

Monocrotaline-induced pulmonary hypertension and right ventricle hypertrophy were further verified to determine right ventricular systolic pressure and RV/LV+S. As shown in Fig. 1, at 3 weeks after the monocrotaline injection, right ventricular systolic pressure was markedly elevated, compared with that in saline-treated animals, in both wild-type and ET_B-deficient *sl*/*sl* groups, and the extent of elevation was greater in *sl*/*sl* rats than in wild-type rats. ABT-627 administration for 3 weeks completely suppressed the monocrotaline-induced elevation of right ventricular systolic pressure in both wild-type and *sl*/*sl* rats. Similar findings were observed in the alterations in RV/LV+S.

3.3. Lung vascular morphology in wild-type and ET_B-deficient homozygous (sl/sl) rats

When lung vascular morphology was evaluated, monocrotaline-treated animals revealed an significant increase in medial thickness of pulmonary arteries with diameters between 50–100 μm and 100–150 μm, compared with cases in saline-treated animals, in both wild-type and ET_B-deficient *sl*/*sl* groups. The extent of medial thickness tended to be enhanced slightly in *sl*/*sl* rats, compared with wild-type animals in each experimental group, although differences were not statistically significant (P=0.136–0.348). Monocrotaline-induced increases in the medial thickness were abolished by the daily administration of ABT-627, in both groups (Fig. 2).

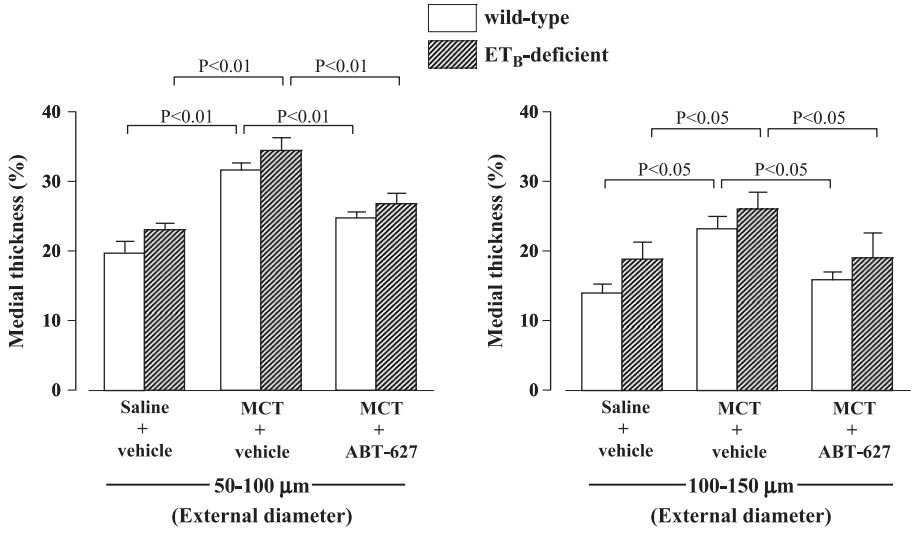

Fig. 2. The effects of ABT-627 on medial wall thickness (%) of small pulmonary arteries in monocrotaline (MCT)-treated wild-type and ET_B-deficient *sl*/*sl* rats 3 weeks after the MCT injection. Each column and bar represent the mean±S.E.M. (without ABT-627, n=10; with ABT-627, n=6).

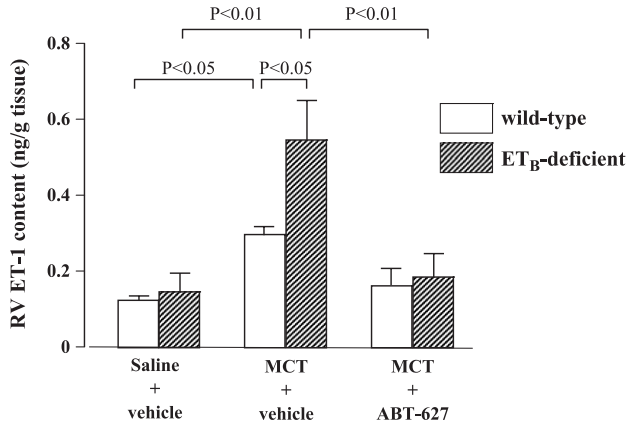

Fig. 3. The effects of ABT-627 on RV ET-1 content of monocrotaline (MCT)-treated wild-type and ET_B-deficient *sl/sl* rats 3 weeks after the MCT injection. Each column and bar represent the mean±S.E.M. (without ABT-627, n=10; with ABT-627, n=6). RV, right ventricle; ET-1, endothelin-1.

3.4. Right ventricle endothelin-1 levels in wild-type and ET_B-deficient homozygous (sl/sl) rats

Endothelin-1 levels in right ventricle tissues are shown in Fig. 3. At 3 weeks after the monocrotaline injection, there was a marked increase in right ventricle endothelin-1 levels, in both wild-type and ET_B-deficient *sl*/*sl* rats, and the levels in *sl*/*sl* rats were significantly high, compared with those in wild-type rats. ABT-627 administration for 3 weeks completely suppressed the monocrotaline-induced increases in right ventricle endothelin-1 levels, in both wild-type and *sl*/*sl* rats.

4. Discussion

Although both selective ET_A and nonselective ET_A/ET_B receptor antagonists have been indicated to suppress the development of pulmonary hypertension induced by monocrotaline treatment (Miyauchi et al., 1993; Hill et al., 1997; Tilton et al., 2000; Jasmin et al., 2001), the role of ET_B receptors in the pathogenesis of pulmonary hypertension of this models is not fully defined. Rats with monocrotaline-induced pulmonary hypertension have a decreased lung expression of ET_B receptors (Yorikane et al., 1993), whereas patients with severe pulmonary hypertension showed an upregulation of ET_B receptor gene (Bauer et al., 2002). Recent studies have suggested that ET_B receptor-mediated actions are protective in pulmonary hypertension, based on findings indicating that chronic ET_B receptor blockade causes pulmonary hypertension in fetal lambs (Ivy et al., 2000) and that hypoxic pulmonary hypertension is exacerbated in ET_B receptor-deficient rats (Ivy et al., 2002). Thus, ET_A-selective antagonists may be superior to non-selective antagonists in the treatment of pulmonary hypertension, because of the beneficial effects of ET_B-stimulation, such as the clearance of endothelin-1 from the circulation (Fukuroda et al., 1994a) and vasorelaxiation through a production of endothelium-derived nitric oxide (Tsukahara et al., 1994).

In monocrotaline-treated rats, the hypotensive effect of endothelin-1 on pulmonary circulation mediated via the ET_B receptor pathway may be enhanced (Sakai et al., 2000). Short-term infusion of RES-701-1, a selective ET_B receptor antagonist, increased pulmonary arterial pressure in beagles with monocrotaline-induced pulmonary hypertension, but not in control animals (Okada et al., 1995). It has been also reported that monocrotaline-treated rats exhibit higher susceptibility to pulmonary vasoconstriction by the treatment with selective ET_B-antagonist BQ-788 compared with the case in saline-treated rats, despite a reduction in ET_B-mediated clearance of endothelin-1 (Dupuis et al., 2000). These findings suggest that ET_B receptor-mediated action plays a protective role through pulmonary vasodilation, against the monocrotaline-induced pulmonary hypertension. Our findings that monocrotaline-induced increases in right ventricular systolic pressure and RV/LV+S, indices of development of pulmonary hypertension, were exaggerated by the genetic deficiency of ET_B receptors, are in agreement with the above view. In addition, marked elevation of right ventricle endothelin-1 level in monocrotaline-treated ET_B receptor-deficient rats appears to be also related to the deteriorative responses in these animals. A previous study have demonstrated the close relationship between monocrotaline-induced pulmonary hypertension and the enhancement of cardiac endothelin-1 gene expression (Miyauchi et al., 1993). Most recently, Jasmin et al. (2003) clearly indicated that monocrotaline-treatment markedly increased the endothelin-1 peptide level in the hypertrophic right ventricle, compared with that in the non-hypertrophic left ventricle. The enhancement of endothelin-1 production in right ventricle of monocrotaline-treated rats seems to be induced by pressure overload to the heart, as suggested by Miyauchi et al. (1993), rather than the primary causal factor of pulmonary hypertension. However, since endothelin-1 is known to induce myocardial cell hypertrophy (Shubeita et al., 1990), a marked increase of right ventricle endothelin-1 induced by monocrotaline may lead to further deterioration of cardiac hypertrophy.

A new finding in the present study is that exaggerated pulmonary hypertension and elevated right ventricle endothelin-1 level in monocrotaline-treated ET_B-deficient rats was completely suppressed by the treatment with ABT-627, a selective ET_A receptor antagonist, to basal levels of wild-type animals. Previously, we demonstrated that enhanced endothelin-1 production and ET_A-mediated actions are responsible for the increased susceptibility to deoxycorticosterone acetate-salt-induced hypertension and tissue injuries in ET_B-deficient rats (Matsumura et al., 2000). Others using salt-loaded rats and cynomolgus monkeys have also proposed that hypertension induced by chronic ET_B receptor blockade is due to the indirect activation of ET_A receptors, based on findings that selective ET_A receptor antagonists abolish the above hypertension (Pollock and Pollock, 2001;

Reinhart et al., 2002). Thus, the antagonism of the ET_A receptors may be essential for the protection from hypertensive diseases, irrespective of the presence of the ET_B receptor-mediated function.

Recently, comparative studies were performed, using a nonselective ET_A/ET_B antagonist BSF420627 and a selective ET_A antagonist LU135252 (Jasmin et al., 2001). Both BSF420627 and LU135252 are effective in rats with monocrotaline-induced pulmonary hypertension, while only BSF420627 significantly reduced right ventricle hypertrophy, suggesting that ET_B receptor-mediated event is associated with the development of hypertrophy and that the nonselective ET_A/ET_B antagonist is superior to the selective ET_A antagonist in the treatment of pulmonary hypertension. However, as the author mentioned, the possibility that differences in the effect of a nonselective ET_A/ET_B antagonist versus a selective ET_A antagonist in the treatment of monocrotaline-induced pulmonary hypertension resulted from differences in the pharmacokinetics and potency of the antagonists used, could not be excluded. Some studies suggest that both ET_A and ET_B blockade are required to inhibit endothelin-1-induced contractions of pulmonary artery (Fukuroda et al., 1994b; Sato et al., 1995; McCulloch et al., 1996). Moreover, it have been reported that both ET_A and ET_B receptors affects the proliferation of pulmonary arterial smooth muscle cells (Davie et al., 2002). On the other hand, we could not demonstrate that ET_B receptor-mediated actions actively play a protective or a causal role in monocrotaline-induced pulmonary hypertension. Further studies using various models of pulmonary hypertension are required to determine whether ET_B receptors play an important role in the pathogenesis of pulmonary hypertension.

The nonselective ET_A/ET_B receptor antagonist bosentan is now approved for the treatment of pulmonary hypertension in humans, based on recent double-blind, placebo-controlled study in patients with pulmonary hypertension, in which bosentan improved pulmonary hemodynamics and exercise capacity (Rubin et al., 2002). In addition, a recent clinical study demonstrated that exercise capacity and cardiopulmonary hemodynamics of pulmonary hypertension patients were also improved by the treatment with sitaxsentan, a selective ET_A antagonist, although this ET_A-selective therapy did not appear to offer any major advantage in terms of safety or efficacy over bosentan (Barst et al., 2004). Thus, the antagonism of the ET_A receptors is essential for the protection from pulmonary hypertension, but further investigation is warranted to determine which type of antagonist is favorable for the treatment of pulmonary hypertension.

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