Endothelin ET$_B$ Receptor Is Involved in Sex Differences in the Development of Balloon Injury-Induced Neointimal Formation

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ABSTRACT

The purpose of this study was to evaluate the involvement of endothelin (ET)$_B$ receptor-mediated action in the sex differences in balloon injury-induced neointimal formation using the spotting-lethal rat, which carries a naturally occurring deletion in its ET$_B$ receptor gene. Male and female ET$_B$-deficient and wild-type rats underwent balloon injury of the carotid artery. In the wild-type rats, the neointima/media ratio was significantly lower in females than in males, but this sex difference was attenuated by ovariectomy and restored by treatment with 17$\beta$-estradiol (20 $\mu$g/kg/day). In the ET$_B$-deficient rats, the neointima/media ratio of the male and female rats was markedly increased to the same level, and this increase was not affected by ovariectomy or 17$\beta$-estradiol treatment. Treatment with (+)-SS,6R,7R)-2-butyl-7-[2-(2S)-2-carboxypropyl]-4-methoxyphenyl]-5-(3,4-methylenedioxyphenyl)cyclopenteno[1,2-b]pyridine-6-carboxylic acid (J-104132) (10 mg/kg/day) markedly decreased the neointima/media ratio to the same extent in males but not intact females. These results indicate that the sex difference in balloon injury-induced neointimal formation was abolished by genetic ET$_B$ receptor deficiency or its pharmacological blockade. The lack of a vasoprotective effect of estrogen and the augmentation of ETA receptor-mediated action seem to be responsible for the abolition of sex differences in the ET$_B$ receptor-inhibited condition.

Introduction

Clinical and epidemiological evidence suggests a sexually dimorphic pattern of atherosclerotic cardiovascular disease in humans. The incidence of cardiovascular disease is lower in women before menopause than in men and postmenopausal women (Bush and Barrett-Connor, 1985; Godsland et al., 1987). These sex differences are considered to be caused by the vasoprotective effect of estrogen (Stampfer et al., 1991; Farhat et al., 1996; Mendelsohn and Karas, 1999). The protective effect of estrogen on the cardiovascular system is closely related to the up-regulation of endothelial nitric oxide (NO) production and the down-regulation of adhesion molecule activity, smooth muscle proliferation/migration, and superoxide production (Tolbert and Oparil, 2001; Miller et al., 2003; Florian et al., 2004). However, the mechanisms behind the sex differences in the incidence of cardiovascular disease and the vascular effect of estrogen have not been fully elucidated. In animal models of vascular lesions such as neointimal formation after vascular injury, it has been reported that male rats develop a more robust neointimal response to vascular injury than females, and neointimal formation is augmented by ovariectomy, and this augmentation is abolished by the vasoprotective effect of estrogen.
by 17β-estradiol (E2) replacement (Chen et al., 1996). However, the mechanisms involved in this sex difference have not been fully elucidated, but are considered to be at least partly related to the vasoprotective actions of estrogen.

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide and has a mitogenic effect on vascular smooth muscle cells (VSMC) (Yanagisawa et al., 1988; Hirata et al., 1989). This peptide is considered to play an important role in the pathophysiology of cardiac, vascular, and renal diseases. The vascular effects of ET-1 are mediated by at least two ET receptor subtypes, ET_A and ET_B. In blood vessels, the ET_A and ET_B receptors are located on VSMC and induce vasoconstriction and cell proliferation. ET_B receptors are expressed not only on VSMC but also on endothelial cells. Endothelial ET_B receptors mediate vasodilative and antiproliferative actions via NO production (Clozel et al., 1992; Winkles et al., 1993; Miyauchi and Masaki, 1999).

The proliferation of VSMC and neointimal formation in response to ET-1 stimulation play key roles in several vascular lesions such as atherosclerosis, restenosis, and arterial hypertrophy caused by hypertension or diabetes (Kirchen-gast and Münter, 1998; Takahashi, 2006). Both selective ET_A receptor and ET_A/ET_B dual receptor antagonists have been indicated to suppress the development of neointimal formation after vascular injury (McKenna et al., 1998; Sammartin et al., 2003; Kitada et al., 2009). Therefore, there is general agreement that the ET-1/ET_A system plays an important role in the development of neointimal formation, although it is disputed which type of antagonist is more effective for the treatment of neointimal formation. Most recently, we have demonstrated that the inhibition of ET_A receptor-mediated actions by its pharmacological blockade or its genetic deficiency leads to an aggravation of neointimal formation after balloon injury (Kitada et al., 2009).

It has been reported that ET-1 systems are involved in the sex differences in the incidence of cardiovascular disease, especially hypertension (Tostes et al., 2008). We have also demonstrated that female rats were more resistant to deoxy-corticosterone acetate salt-induced hypertension than male rats and this sex difference was abolished by genetic deficiency of the ET_A receptor (Kawanishi et al., 2007). Thus, ET_A receptor-mediated actions seem to contribute to the sex differences in the development of cardiovascular diseases.

The purpose of the present study was to evaluate the involvement of ET_B receptor-mediated action in the sex differences observed in the incidence of balloon injury-induced neointimal formation in the spotting-lethal (sl/sl) rat, which carries a naturally occurring deletion in its ET_B receptor gene. Because homozygous (sl/sl) rats do not live beyond 1 month of age because of intestinal aganglionosis and the resulting intestinal obstruction, the dopamine β-hydroxylase promoter was used to direct ET_B transgene expression in sl/sl rats to support normal enteric nervous system development. These transgenic sl/sl rats live into adulthood and are healthy, expressing ET_B receptors in their adrenal glands and other adrenergic neurons. However, they are ET_B-deficient in other tissues, with their most important characteristic being ET_B receptor deficiency in the vascular endothelium and vascular smooth muscle (Gariepy et al., 1998, 2000). This ET_B receptor-deficient rat is a useful tool for determining the pathophysiological roles of ET_B receptors in vascular tissues.

### Materials and Methods

**Animals.** Two series of experiments were carried out. In the first series, male and female ET_B receptor-deficient (sl/sl) and wild-type (+/+) rats (12–15 weeks of age) were used. In the second series, to investigate the vasoprotective effects of ET receptor antagonists, male and female Sprague-Dawley (SD) rats (10 balloon injury) (Japan SLC, Shizuoka, Japan) were used. The creation of transgenic sl/sl rats has been described previously (Gariepy et al., 1998). Homozygous (sl/sl) rats have dark eyes and small spots on their heads. Wild-type and heterozygous rats have pigmented heads, backs, and tails. To definitively differentiate these rats, polymerase chain reaction (PCR) was performed on DNA isolated from tail biopsy specimens as described previously (Gariepy et al., 1998). The animals were housed in a light-controlled room under a 12-h light/dark cycle and allowed ad libitum access to food and water. All experimental protocols and animal care methods were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences.

**Experimental Protocol.** Female ET_B-deficient and wild-type rats were divided into three groups: the intact female, ovariectomy (OVX), and OVX + E2 groups. Under anesthesia, which was achieved using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), female ET_B-deficient and wild-type rats were subjected to OVX or sham surgery. A week later, daily subcutaneous administration of E2 (20 μg/kg/day) or vehicle was started. After 3 days of E2 administration, the rats were subjected to balloon injury of the right carotid artery. The administration of E2 or vehicle was continued until 2 weeks after the balloon injury. Furthermore, some of the intact female, male ET_B-deficient, and wild-type rats were gavaged with (+)-5S,6R,7R)-2-butyl-7-[2-((2S)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylenedioxyphenoxy)cyclopenteno[1,2-b]pyri
dine-6-carboxylic acid (J-104132) (an ET_A/ET_B dual receptor antagonist; 10 mg/kg/day) (Nishikibe et al., 1999) for 2 weeks, starting 12 h after the balloon injury. SD rats (10 weeks of age) were used for the balloon injury procedure. After the balloon injury, the SD rats were divided into groups treated by: 2R-(4-propoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-(N-(2,6-diethylphenyl)aminocarbonyl-methyl)-pyrrolidine-3R-carboxylic acid (A-192621) (a selective ET_A receptor antagonist; 30 mg/kg/day), 2R-(4-methoxyphenyl)-4S-(1,3-benzodi
oxol-5-yl)-1-(N,N-di-n-butylaminocarbonyl-methyl)pyrrolidine-3R-carboxylic acid (ABT-627) (a selective ET_A receptor antagonist; 10 mg/kg/day), J-104132 (an ET_A/ET_B dual receptor antagonist; 10 mg/kg/day), and J-104132 (an ET_A/ET_B dual receptor antagonist; 10 mg/kg/day). The rats were then gavaged with vehicle, A-192621, ABT-627, or J-104132 for 2 weeks, starting 12 h after the balloon injury. These doses of A-192621, ABT-627, and J-104132 have been shown to almost abolish endogenous ET-1-induced neointimal formation (Kitada et al., 2009). In all animals, 2 weeks after the balloon injury, systolic blood pressure (SBP) was measured by the tail-cuff method using a pneumatic pulse trans
cducer (BP-98A; Sofrtron, Tokyo, Japan).

**Balloon Injury Procedure.** The rats were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), and the right carotid artery was injured with a 2F Fogarty balloon catheter (Baxter International, Deerfield, IL), as described previously (Mori et al., 2000). The left carotid artery was not damaged. Two weeks after the balloon injury, the rats were sacrificed with a sodium pentobarbital overdose (75 mg/kg), and the left and right carotid arteries were harvested. Uteri were also isolated and weighed to assess the effects of ovariectomy and 17β-estradiol supplementation.

**Morphometric Analysis.** The bilateral carotid arteries were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm-thick sections. The tissue sections were then stained by the Elastica Van Gieson method. Morphometric analysis of each arterial segment was performed with a computer-based Motic Image Plus 2.0 Morphomet-

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measured to assess the neointimal and medial areas. The ratio of neointimal-to-medial area (neointima/media ratio) was calculated by dividing neointimal area by medial area.

**Plasma ET-1 Level.** In separate experiments, ET<sub>B</sub>-deficient, wild-type rats and SD rats treated with vehicle, A-192621, ABT-627, and J-104132 were anesthetized with sodium pentobarbital (50 mg/day), and blood was withdrawn from the abdominal aorta for analysis. The plasma ET-1 level was analyzed using an enzyme immunoassay kit (Assay Designs, Ann Arbor, MI).

**Total RNA Extraction, Reverse Transcription, and Real-Time PCR.** Total RNA was isolated from the uninjured and injured carotid artery using RNAiso Plus (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Real-time reverse transcription-PCR analysis was performed using the SYBR PrimeScript reverse transcription-PCR kit (Takara Bio Inc.) and CFX96 (Bio-Rad Laboratories, Tokyo, Japan). The rat ET<sub>B</sub>, ET<sub>A</sub>, and GAPDH primer sequences were as follows: forward primer, 5'-CAGGGAGCGAGCCACCAACACA-3’ (ET<sub>B</sub>), 5'-GATACGCAAAGGTCTCCGCTCCA-3' (ET<sub>A</sub>), 5’-CGGTGTGAAAGGATTTGG-3’ (GAPDH); reverse primer, 5’-TGCTGCGAGGCACGTTCACA-3’ (ET<sub>B</sub>), 5’-GTCCACAGGACAAAGAGAAGA-3’ (ET<sub>A</sub>), 5’-TGAAGGGGTGTGGATGG-3’ (GAPDH). Comparisons with GAPDH values were carried out for normalization. The relative expression levels in each sample were determined by comparisons with the standard using Bio-Rad CFX Manager Software for analysis.

**Drugs.** 17β-Estradiol was obtained from Nakalai Tesque (Kyoto, Japan) and dissolved in cottonseed oil. A-192621 was provided by Abbott Laboratories (Abbott Park, IL) and dissolved in 0.02 N NaOH. ABT-627 was provided by Abbott Laboratories and dissolved in a mixture of 10% ethanol, 40% propylene glycol, and 50% distilled water. J-104132 was provided by Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan) and dissolved in distilled water. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), Nakalai Tesque (Kyoto, Japan), and Wako Pure Chemicals (Osaka, Japan).

**Statistical Analysis.** All values are expressed as the mean ± S.E.M. Relevant data were processed using InStat (GraphPad Software, Inc., San Diego, CA). For statistical analysis, we used one-way analysis of variance followed by Bonferroni's or Dunnett's multiple comparison tests. Differences were considered significant at *P* < 0.05.

**Results**

**Body Weight, Uterus Wet Weight, and Systolic Blood Pressure.** Two weeks after the balloon injury, the body weight of the intact females was significantly lower than that of the male rats in both the wild-type and ET<sub>B</sub>-deficient rats. In both the female wild-type and ET<sub>B</sub>-deficient rats, body weight was significantly increased in the OVX rats compared with that of the intact female rats and was restored to the level of the intact females in the OVX + E<sub>2</sub> rats (Table 1).

The wet weight of the uteri of the OVX rats was significantly decreased compared with that of the intact female rats and was restored to the level of the intact females in both the wild-type and ET<sub>B</sub>-deficient OVX + E<sub>2</sub> rats. There were no significant differences between the wild-type and ET<sub>B</sub>-deficient rats in body weight or uteri wet weight (Table 1).

The SBP of the ET<sub>B</sub>-deficient rats was significantly higher than that of the wild-type rats in each group. No significant difference in SBP was found among the male, female, OVX, or OVX + E<sub>2</sub> group in either genotype (Table 1).

**Effects of ET<sub>B</sub> Receptor Deficiency and Estrogen on Neointimal Formation.** In the uninjured arteries, neointimal formation was not observed in any animal (data not shown). In the injured arteries, neointimal thickening was observed. In the wild-type rats, the extent of neointimal formation was more marked in the male rats than in the female rats (Fig. 1, A and B; Table 1). Neointimal formation was markedly increased in the OVX rats, and this increase in neointimal formation was markedly improved in the OVX + E<sub>2</sub> rats (Fig. 1, C and D; Table 1). In the ET<sub>B</sub>-deficient rats, neointimal formation in the male, intact female, OVX, and OVX + E<sub>2</sub> rats was markedly increased to the same extent (Fig. 1, E-H; Table 1). Figure 2 shows the results of morphometric analysis of the injured arteries. In the wild-type rats, the neointima/media ratio of the male rats was significantly higher than that of the intact female rats (Fig. 2). Compared with the intact female rats, the neointima/media ratio of the OVX rats was significantly increased. The increased neointima/media ratio in the OVX rats was significantly improved in the OVX + E<sub>2</sub> rats (Fig. 2). In the ET<sub>B</sub>-deficient rats, the neointima/media ratio was markedly and similarly increased in the male, intact female, OVX, and OVX + E<sub>2</sub> rats (Fig. 2).

**Effect of J-104132 on Neointimal Formation in ET<sub>B</sub>-Deficient Rats.** Treatment with J-104132 for 2 weeks after the balloon injury did not affect body weight, uteri wet weight, or SBP (data not shown). In the male rats, 2-week treatment with J-104132 markedly decreased the neointima/media ratio in both the wild-type and ET<sub>B</sub>-deficient rats. In the female rats, treatment with J-104132 markedly decreased the neointima/media ratio in the ET<sub>B</sub>-deficient rats but not in the wild-type rats (Fig. 3).

**Neointimal Formation after Balloon Injury and Effects of Pharmacological Blockade in SD Rats.** Treatment with

**Table 1** Comparative data on body weight, uteri wet weight, systolic blood pressure, and neointima and media area

<table>
<thead>
<tr>
<th></th>
<th>Body Weight</th>
<th>Uteri Wet Weight</th>
<th>SBP</th>
<th>Area of Neointima</th>
<th>Area of Media</th>
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<tr>
<td></td>
<td>g</td>
<td>mmHg</td>
<td>mm&lt;sup&gt;2&lt;/sup&gt;</td>
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<td><strong>Wild type</strong></td>
<td></td>
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<tr>
<td>Male</td>
<td>370 ± 12&lt;sup&gt;**&lt;/sup&gt;</td>
<td>127 ± 3</td>
<td>0.140 ± 0.004&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.128 ± 0.003&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Female</td>
<td>253 ± 2</td>
<td>116 ± 4</td>
<td>0.063 ± 0.009</td>
<td>0.103 ± 0.002</td>
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<tr>
<td>OVX</td>
<td>283 ± 7</td>
<td>120 ± 4</td>
<td>0.120 ± 0.016</td>
<td>0.105 ± 0.003</td>
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<tr>
<td>OVX + E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>258 ± 3</td>
<td>117 ± 2</td>
<td>0.073 ± 0.004</td>
<td>0.106 ± 0.003</td>
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<tr>
<td><strong>ET&lt;sub&gt;B&lt;/sub&gt;-deficient</strong></td>
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<tr>
<td>Male</td>
<td>368 ± 8&lt;sup&gt;**&lt;/sup&gt;</td>
<td>141 ± 3</td>
<td>0.214 ± 0.016&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.137 ± 0.004&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Female</td>
<td>246 ± 4</td>
<td>140 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.158 ± 0.006&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.103 ± 0.004</td>
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<tr>
<td>OVX</td>
<td>279 ± 4&lt;sup&gt;**&lt;/sup&gt;</td>
<td>138 ± 1&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.178 ± 0.017&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.130 ± 0.009&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>OVX + E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>254 ± 4</td>
<td>138 ± 2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.177 ± 0.013&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.123 ± 0.005&lt;sup&gt;**&lt;/sup&gt;</td>
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<sup>*P* < 0.05, <sup>**</sup>*P* < 0.01 compared with the females of the same genotype group.  
<sup>†</sup>*P* < 0.05, <sup>††</sup>*P* < 0.01 compared with the OVX rats of the same genotype group.  
<sup>‡</sup>*P* < 0.05, <sup>‡‡</sup>*P* < 0.01 compared with corresponding wild-type rats.
A-192621, ABT-627 and J-104132 for 2 weeks after the balloon injury did not affect body weight, uteri wet weight, or SBP (data not shown).

The neointima/media ratio of the vehicle-treated female rats was significantly lower than that of male rats, and this sex difference was abolished by A-192621 treatment (Fig. 4). In the male rats, 2 weeks of treatment with ABT-627 or J-104132 significantly decreased the neointima/media ratio to the same extent. In the female rats, treatment with ABT-627 or J-104132 did not affect the neointima/media ratio after the balloon injury (Fig. 4).

**Plasma ET-1 Level.** Compared with the wild-type rats, the ET$_B$-deficient rats exhibited significantly increased plasma ET-1 levels, in both males and females. There were no significant differences between the male and female rats in either genotype (Fig. 5).

In the female SD rats, A-192621 or J-104132 treatment significantly increased plasma ET-1 levels compared with vehicle-treated group (9.35 ± 0.15 pg/ml versus 0.38 ± 0.15 pg/ml, respectively; p < 0.01). Meanwhile, ABT-627-treated female rats exhibited no significant differences of plasma ET-1 levels by ET receptor antagonists were similar to those observed in male rats. Plasma ET-1 levels of male rats and the effects of ET receptor antagonists were reported in our previous study (Kitada et al., 2009).

**Effects of ET$_B$ Receptor Deficiency and Estrogen on Vascular ET$_A$ and ET$_B$ Receptor mRNA Expression after Balloon Injury.** No significant differences in ET$_A$ or ET$_B$ receptor mRNA expression were found among any of the groups in the uninjured or injured carotid arteries 2 weeks after the balloon injury (data not shown).
Sex Differences in Neointimal Formation and ET<sub>B</sub> Receptor

Fig. 4. Neointima/media ratio of the injured arteries in SD rats at 2 weeks after balloon injury and the effects of A-192621, ABT627, and J-104132. Data are expressed as the mean ± S.E.M. (n = 4–5). **, P < 0.01, compared with the vehicle-treated male rat group. ††, P < 0.01, compared with the vehicle-treated female rat group.

Fig. 5. Plasma ET-1 levels of wild-type (left) and ET<sub>B</sub>-deficient (right) rats. Data are expressed as the mean ± S.E.M. (n = 4). **, P < 0.01, compared with wild-type rats of the same sex.

Discussion

It has been indicated that the ET-1 system is involved in the sex differences observed in the frequency of cardiovascular disease (Tostes et al., 2008). In rat hypertension models, ET<sub>B</sub> receptor-mediated actions were reported to play an important role in the sex differences in salt-induced hypertension (Taylor et al., 2003; Sullivan et al., 2006; Kawanishi et al., 2007). However, it has not been revealed whether ET<sub>B</sub> receptor-mediated actions are involved in the sex differences of vascular lesions. In the present study, the frequency of neointimal formation after balloon injury in wild-type rats was much lower in intact females than in males. In contrast, in ET<sub>B</sub>-deficient rats, the incidence of neointimal formation after balloon injury was markedly increased to the same extent in the males and intact females, indicating that the sex differences in this vascular lesion were abolished by genetic ET<sub>B</sub> receptor deficiency. Furthermore, A-192621, a selective ET<sub>B</sub> receptor antagonist, abolished the sex differences of balloon injury-induced neointimal formation in SD rats. These findings suggest that the ET<sub>B</sub> receptor plays an important role in the sex differences observed in the development of balloon injury-induced neointimal formation.

The vasoprotective effects of estrogen are well established in humans and experimental animals, although the precise molecular mechanisms behind them have not been fully elucidated (Stampfer et al., 1991; Farhat et al., 1996; Mendelsohn and Karas, 1999; Xing et al., 2009). It is well known that postmenopausal women who receive estrogen replacement therapy (ERT) have a substantially lower risk of cardiovascular disease (Stampfer et al., 1991; Walsh et al., 1991; Grady et al., 1992). However, the Heart Estrogen-Progestin Replacement Study and the Women's Health Initiative Clinical Trial and observational study did not show any benefit of ERT (Hulley et al., 1998; Rossow et al., 2002). Thus, the effect of ERT on cardiovascular disease is still disputed. Therefore, determination of the mechanisms of estrogen-induced vasoprotective effects remains a critical issue. In this study, neointimal formation after vascular injury in female wild-type rats was significantly aggravated by OVX, and this aggravation was markedly improved by E<sub>2</sub> treatment, clearly indicating that estrogen inhibits neointimal formation after vascular injury in wild-type rats. Of particular interest is that OVX and E<sub>2</sub> treatment failed to affect the enhanced neointimal formation observed in female ET<sub>B</sub>-deficient rats. In both female wild-type and ET<sub>B</sub>-deficient rats, a significant increase in body weight and a decrease in uterine weight were observed in the OVX group. These OVX-induced changes were almost completely restored by E<sub>2</sub> treatment to the level seen in the intact females, indicating that OVX and E<sub>2</sub> treatments are appropriate for assessing the effect of estrogen. Because the vasoprotective effects of estrogen after vascular injury were abolished by genetic deficiency of the ET<sub>B</sub> receptor, estrogen is likely to reduce neointimal formation after vascular injury via a mechanism that depends on ET<sub>B</sub> receptor-mediated action, and the ET<sub>B</sub> receptor-mediated action seems to occur downstream of the vasoprotective effects of estrogen. Alternatively, the possibility that marked augmentation of balloon injury-induced neointimal formation by ET<sub>B</sub> receptor deficiency produces functional abolition of the abovementioned sex differences cannot be ruled out. Further investigations are required to clarify the cross-talk between estrogen receptor- and ET<sub>B</sub> receptor-mediated actions.

It has been reported that there are sex differences in vascular ET-1 receptor density and that estrogen modulates vascular ET-1 receptor expression. In human saphenous veins, men exhibit an increased number of ET-1 receptors and an increased ratio of ET<sub>A</sub> to ET<sub>B</sub> receptors compared with women (Ergul et al., 1998). In an animal study, 17β-estradiol induced up-regulation of ET<sub>B</sub> receptor gene expression in rabbit coronary arteries (Pedersen et al., 2008). In deoxycorticosterone acetate-salt-induced hypertension rats, vascular mRNA expression of ET<sub>B</sub> receptors was increased in males compared with that observed in females (David et al., 2001, 2002). Taken together, it seems likely that estrogen modulates vascular ET-1 receptor expression, mainly ET<sub>B</sub> receptor expression. On the other hand, it has been reported that the expression levels of ET-1, endothelin-converting enzyme, and the ET<sub>A</sub> and ET<sub>B</sub> receptors are increased in rat carotid arteries after balloon injury (Wang et al., 1996). Therefore, alternations in ET-1 receptor expression induced by estrogen may be at least partly involved in the sex differences in the frequency of balloon injury-induced neointimal formation. In the present study, we estimated the mRNA levels of the ET<sub>A</sub> and ET<sub>B</sub> receptors in balloon-injured arter-
ies. However, there were no significant differences in ET$_A$ or ET$_B$ receptor mRNA levels among the groups. Furthermore, in a separate study, we examined ET$_A$ and ET$_B$ receptor expression in injured arteries from male and female wild-type rats by immunohistochemistry, but there were no significant differences in ET$_A$ or ET$_B$ receptor expression between male and female rats (data not shown). Thus, the modulation of ET-1 receptor expression by estrogen in injured arteries after vascular injury does not seem to contribute to the sex differences in balloon injury-induced neointimal formation. However, we did not analyze their time-dependent changes. Because there has been no report on the sex differences in ET-1 receptor expression in injured arteries, further evaluation of ET-1 receptor expression after vascular injury is an important issue.

It is acknowledged that ET$_B$ receptors are responsible for the clearance of ET-1 from the circulation (Fukuroda et al., 1994), and plasma ET-1 levels are known to increase in ET$_B$-deficient rats (Taylor et al., 2003; Sullivan et al., 2006). In the present study, we measured the plasma ET-1 levels in male and female ET$_B$-deficient and wild-type rats. The plasma ET-1 level of the ET$_B$-deficient rats was markedly increased compared with that of the wild-type rats in both the male and female rats. In the wild-type rats, the plasma ET-1 level was comparable in male and female rats, whereas the incidence of neointimal formation after balloon injury in female rats was reduced compared with that for male rats. On the other hand, in ET$_B$-deficient rats, both the plasma ET-1 level and neointimal formation after balloon injury were comparable in male and female rats. In addition, we examined the mRNA level of ET-1 in the injured arteries, but there were no significant differences between male and female rats in the wild-type or ET$_B$-deficient groups. Furthermore, we examined the plasma ET-1 levels in ET antagonist-treated rats. A-192621 treatment significantly increased the plasma ET-1 levels in both male and female rats, but there were no significant differences between vehicle- or A-192621-treated male and female rats. Although we could not directly evaluate the concentration and localization of ET-1 in the injured arteries, the above findings suggest that ET$_B$ receptor-mediated action rather than ET-1 clearance via the ET$_B$ receptor is related to the sex differences in balloon injury-induced neointimal formation.

In cardiovascular disease animal models, such as salt-induced hypertensive rats and monocrotaline-induced pulmonary hypertensive rats, the development of disease is aggravated by the chronic inhibition of ET$_B$ receptors, and this aggravation can be abolished using an ET$_A$ receptor antagonist (Matsumura et al., 2000; Nishida et al., 2004). These findings suggest that chronic inhibition of ET$_B$ receptors leads to the overstimulation of ET$_A$ receptors and that the aggravation of disease under ET$_B$ receptor-inhibited conditions is prevented by the blockade of the ET$_A$ receptor. In the present study, ABT-627 and J-104132 markedly decreased neointima/media ratio to the same extent in male SD rats. On the other hand, treatment with ABT-627 or J-104132 did not affect neointimal formation after balloon injury in female SD rats. Consequently, these results indicate that there are sex differences in the vasoprotective effects of ET receptor antagonists and that ET$_A$-induced neointimal formation after balloon injury in intact female rats is reduced compared with that observed in male rats. Moreover, the aggravation of neointimal formation observed under ET$_B$ receptor-inhibited conditions was not observed in the ET$_A$ receptor-inhibited conditions. Meanwhile, treatment with J-104132, an ET$_A$/ET$_B$ dual receptor antagonist, markedly decreased neointimal formation after balloon injury in female ET$_B$-deficient rats. Thus, it seems likely that the augmentation of ET$_A$ receptor-mediated ET-1 action mainly contributes to the enhancement of neointimal formation observed in female ET$_B$-deficient rats. These results indicate that the augmentation of ET$_A$ receptor-mediated action rather than ET$_B$ receptor deficiency itself contributes to the abolition of the sex differences in ET$_B$-deficient rats.

In conclusion, ET$_B$ receptor-mediated action is involved in the differences in the incidence of balloon injury-induced neointimal formation between male and female rats, although the relationship between the ET$_B$ receptor- and estrogen receptor-signaling systems remains unclear. In addition, the lack of the vasoprotective effects of estrogen and the augmentation of ET$_A$ receptor-mediated action seem to be responsible for the abolition of sex differences in ET$_B$ receptor deficiency.

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References


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