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

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A list of nonstandard abbreviations: NO, nitric oxide; ET-1, endothelin-1; VSMC, vascular smooth muscle cells; DOCA, deoxycorticosterone acetate; sl, spotting-lethal; OVX, ovariectomy; 17$\beta$-estradiol, E$_2$; SBP, systolic blood pressure.

Recommended section: Cardiovascular
Abstract

The purpose of this study was to evaluate the involvement of 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 17β-estradiol (20 μg/kg/day) treatment. 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 17β-estradiol treatment. Treatment with J-104132 (10 mg/kg/day), an ET_A/ET_B dual receptor antagonist, markedly decreased the neointima/media ratio of the male wild-type rats and the male and female ET_B-deficient rats, but not the female wild-type rats. In addition, A-192621 (30 mg/kg/day), a selective ET_B receptor antagonist, abolished the sex difference of balloon injury-induced neointimal formation. ABT-627 (10 mg/kg/day), a selective ET_A receptor antagonist, and 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 ET_A 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 prior to the menopause than in men and postmenopausal women (Godsland et al., 1987; Bush and Barrett-Connor, 1985). These sex differences are considered to be due to 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 upregulation of endothelial nitric oxide (NO) production and the downregulation of adhesion molecule activity, smooth muscle proliferation/migration, and superoxide production (Florian et al., 2004; Miller et al., 2003; Tolbert and Oparil, 2001). 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 that neointimal formation is augmented by ovariectomy and that this augmentation is abolished by 17β-estradiol 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, i.e., the ET_A and ET_B receptors. In blood vessels, the ET_A and ET_B receptors are located on VSMC and induce vasoconstriction and cell
proliferation. ET<sub>B</sub> receptors are expressed not only on VSMC but also on endothelial cells. Endothelial ET<sub>B</sub> receptors mediate vasodilative and antiproliferative actions via NO production (Winkles et al., 1993; Miyauchi and Masaki, 1999; Clozel et al., 1992).

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 due to hypertension or diabetes (Kirchengast and Munter, 1998; Takahashi, 2006). Both selective ET<sub>A</sub> receptor and ET<sub>A</sub>/ET<sub>B</sub> dual receptor antagonists have been indicated to suppress the development of neointimal formation after vascular injury (McKenna et al., 1998; Sanmartin et al., 2003; Kitada et al., 2009). Therefore, there is general agreement that the ET-1/ET<sub>A</sub> 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<sub>B</sub> receptor-mediated actions by its pharmacological blockade or by 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, and especially hypertension (Tostes et al., 2008). We have also demonstrated that female rats were more resistant to deoxycorticosterone acetate (DOCA)-salt-induced hypertension than male rats and that this sex difference was abolished by genetic deficiency of the ET<sub>B</sub> receptor (Kawanishi et al., 2007). Thus, ET<sub>B</sub> 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<sub>B</sub> receptor-mediated action in the sex differences observed in the incidence of balloon
injury-induced neointimal formation using the spotting-lethal (sl) rat, which carries a naturally occurring deletion in its ET\textsubscript{B} receptor gene. As homozygous (sl/sl) rats do not live beyond 1 month of age due to intestinal aganglionosis and the resulting intestinal obstruction, the dopamine \( \beta \)-hydroxylase (DBH) promoter was used to direct ET\textsubscript{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\textsubscript{B} receptors in their adrenal glands and other adrenergic neurons. However, they are ET\textsubscript{B}-deficient in other tissues, with their most important characteristic being ET\textsubscript{B} receptor deficiency in the vascular endothelium and vascular smooth muscle (Gariepy et al., 1998, 2000). This ET\textsubscript{B} receptor-deficient rat is a useful tool for determining the pathophysiological roles of ET\textsubscript{B} receptors in vascular tissues.
Materials and Methods

Animals  Two series of experiments were carried out. In the first series, male and female ETB 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 weeks of age) (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, the polymerase chain reaction 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-hour light/dark cycle and were 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 ETB-deficient and wild-type rats were divided into 3 groups, the intact female, ovariectomy (OVX), and OVX + 17β-estradiol (E2) groups. Under anesthesia, which was achieved using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), female ETB-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 treatment, 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 ETB-deficient, and wild-type rats were gavaged with J-104132 (an ET₃/ETB dual receptor antagonist: 10 mg/kg/day) (Nishikibe et al., 1999) for 2 weeks, starting 12 hours 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 vehicle-treated, A-192621 (a selective ET<sub>B</sub> receptor antagonist: 30 mg/kg/day)-treated, ABT-627 (atrasentan; a selective ET<sub>A</sub> receptor antagonist: 10 mg/kg/day)-treated, and J-104132 (an ET<sub>A</sub>/ET<sub>B</sub> dual receptor antagonist: 10 mg/kg/day)-treated groups. The rats were then gavaged with vehicle, A-192621, ABT-627, or J-104132 for two weeks, starting 12-h after the balloon injury. These doses of A-192621, ABT-627 and J-104132 have previously 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 transducer (BP-98A; Softron, 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, USA), 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 Morphometric system (Shimadzu, Kyoto). The borders of the lumen, internal elastic lamina, and external elastic lamina were traced and 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\textsubscript{B}-deficient, wild-type rats, vehicle-treated, A-192621-treated, ABT-627-treated and J-104132- treated SD rats 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, Inc. 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 RT-PCR analysis was performed using the SYBR\textsuperscript{®} PrimeScript\textsuperscript{®} RT-PCR Kit (Takara Bio Inc.) and CFX96\textsuperscript{TM} (Bio-Rad Laboratories, Inc., Tokyo, Japan). The rat ET\textsubscript{A}, ET\textsubscript{B}, and GAPDH primer sequences were as follows: forward primer, 5’-CAGGAGCAGAACCACAACACA-3’ (ET\textsubscript{A}), 5’-GATACGACAACTTCCGCTCCA-3’ (ET\textsubscript{B}), 5’-CGGTGTGAACGGATTTGG-3’ (GAPDH); reverse primer, 5’-TGCTGCGTGACCGTTTCA-3’ (ET\textsubscript{A}), 5’-GTCCACGATGAGGACAATGAGA-3’ (ET\textsubscript{B}), 5’-TGAAGGGGTCGTTGATGGG-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 the Bio-Rad CFX Manager Software for analysis.

**Drugs** 17\(\beta\)-Estradiol was obtained from Nakalai Tesque (Kyoto, Japan) and dissolved in cottonseed oil. A-192621 [2\(R\)-(4-propoxyphenyl)-4\(S\)-(1,3-benzodioxol-5-yl)-1-(N-(2,6-diethylphenyl)aminocarbonyl-methyl)-pyrrolidine-3\(R\)-carboxylic acid] was provided by Abbott Laboratories (Abbott Park, IL) and dissolved in a 0.02N NaOH. ABT-627
[2R-(4-methoxyphenyl)-4S-(1,3-benzodioxol-5-yl)-1-(N,N-di(n-butyl)aminocarbonyl-methyl)-pyrrolidine-3R-carboxylic acid] was provided by Abbott Laboratories (Abbott Park, IL) and dissolved in a mixture of 10% ethanol, 40% propylene glycol, and 50% distilled water. J-104132

[(+)-(5S,6R,7R)-2-butyl-7-[2-((2S)-2-carboxypropyl)-4-methoxyphenyl]-5-(3,4-methylenedioxyphenyl)cyclopenteno[1,2-b]pyridine-6-carboxylic acid] was provided by Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan) and dissolved in distilled water. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Nakalai Tesque (Kyoto, Japan), and Wako (Osaka, Japan).

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

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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$_B$-deficient rats. In both the female wild-type and ET$_B$-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$_2$ rats (Table).

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$_B$-deficient OVX + E$_2$ rats. There were no significant differences between the wild-type and ET$_B$-deficient rats in body weight or uteri wet weight (Table).

The SBP of the ET$_B$-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$_2$ group in either genotype (Table).

Effects of ET$_B$-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 (Figures 1A and 1B, Table). Neointimal formation was markedly increased in the OVX rats, and this increase in neointimal formation was markedly improved in the OVX + E$_2$ rats (Figures 1C and 1D, Table). In the ET$_B$-deficient rats, neointimal formation in the male, intact female, OVX, and OVX + E$_2$ rats was markedly increased to the same extent (Figures 1E-1H, Table). 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 (Figure 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 + E2 rats (Figure 2). In the ETB-deficient rats, the neointima/media ratio was markedly and similarly increased in the male, intact female, OVX, and OVX + E2 rats (Figure 2).

**Effect of J-104132 on Neointimal Formation in ETB-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-weeks treatment with J-104132 markedly decreased the neointima/media ratio in both the wild-type and ETB-deficient rats. In the female rats, treatment with J-104132 markedly decreased the neointima/media ratio in the ETB-deficient rats but not in the wild-type rats (Figure 4).

**Neointimal Formation after Balloon Injury and Effects of Pharmacological Blockade in SD Rats** Treatment with 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 significantly lower than that of male rats, and this sex difference was abolished by A-192621 treatment (Figure 5). In the male rats, 2 weeks 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 (Figure 5).

**Plasma ET-1 Level** Compared with the wild-type rats, the ETB-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.
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 or 23.70 ± 1.82 pg/ml vs. 0.38 ± 0.15 pg/ml, respectively, p<0.01). Meanwhile, ABT-627-treated female rats exhibited no significant differences of plasma ET-1 level compared with vehicle-treated rats (2.00 ± 0.62 pg/ml vs. 0.38 ± 0.15 pg/ml). In addition, these changes and values 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 have already reported in our previous study (Kitada et al., 2009).

**Effects of ETB-Receptor Deficiency and Estrogen on Vascular ETA and ETB Receptor mRNA Expression after Balloon Injury.** No significant differences in ETA or ETB 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).
Discussion

It has recently been indicated that the ET-1 system is involved in the sex differences observed in the frequency of cardiovascular disease (Tostes et al., 2008). Using rat hypertension models, ET_B receptor-mediated actions were reported to play an important role in the sex differences in salt-induced hypertension (Kawanishi et al., 2007; Taylor et al., 2003; Sullivan et al., 2006). However, it has not been revealed whether ET_B 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_B-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_B receptor deficiency. Furthermore, A-192621, a selective ET_B receptor antagonist, abolished the sex differences of balloon injury-induced neointimal formation in SD rats. These findings suggest that the ET_B 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; Grady et al., 1992; Walsh et al., 1991). However, the Heart Estrogen-Progestin Replacement Study (HERS) and the Women’s Health Initiative Clinical Trial and observational study (WHI) 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 E2 treatment, clearly indicating that estrogen inhibits neointimal formation after vascular injury in wild-type rats. Of particular interest is that OVX and E2 treatment failed to affect the enhanced neointimal formation observed in female ETB-deficient rats. In both female wild-type and ETB-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 E2 treatment to the level seen in the intact females, indicating that OVX and E2 treatments are appropriate for assessing the effect of estrogen. Since the vasoprotective effects of estrogen after vascular injury were abolished by genetic deficiency of the ETB receptor, estrogen is likely to reduce neointimal formation after vascular injury via a mechanism that is dependent on ETB receptor-mediated action, and the ETB 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 ETB receptor deficiency produces functional abolition of the abovementioned sex differences cannot be ruled out. Further investigations are required to clarify the crosstalk between estrogen receptor- and ETB 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 as well as an increased ratio of ETα to ETβ receptors compared with women (Ergul et al., 1998). In an animal study, 17β-estradiol induced upregulation of ETβ receptor gene expression in rabbit
coronary arteries (Pedersen et al., 2008). In DOCA-salt-induced hypertension rats, vascular mRNA expression of ET_B receptors is 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_B receptor expression. On the other hand, it has been reported that the expression levels of ET-1, endothelin converting enzyme, and the ET_A and ET_B 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_A and ET_B receptors in balloon-injured arteries. However, there were no significant differences in ET_A or ET_B receptor mRNA levels among each group. 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. Since 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
The plasma ET-1 level of the ETB-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 the ETB-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 ETB-deficient group. Furthermore, we examined the plasma ET-1 levels in ET antagonist-treated rats. Actually, 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 ETB receptor-mediated action rather than ET-1 clearance via the ETB 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 ETB receptors, and this aggravation can be abolished using an ETA receptor antagonist (Matsumura et al., 2000; Nishida et al., 2004). These findings suggest that chronic inhibition of ETB receptors leads to the overstimulation of ETA receptors and that the aggravation of disease under ETB receptor-inhibited conditions is prevented by the blockade of the ETA receptor. In the present study, ABT-627 and J-104132 markedly decreased the 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\textsubscript{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\textsubscript{B} receptor-inhibited conditions was not observed in the ET\textsubscript{A} receptor-inhibited conditions. Meanwhile, treatment with J-104132, an ET\textsubscript{A}/ET\textsubscript{B} dual receptor antagonist, markedly decreased neointimal formation after balloon injury in female ET\textsubscript{B}-deficient rats. Thus, it seems likely that the augmentation of ET\textsubscript{A} receptor-mediated ET-1 action mainly contributes to the enhancement of neointimal formation observed in female ET\textsubscript{B}-deficient rats. These results indicate that the augmentation of ET\textsubscript{A} receptor-mediated action rather than ET\textsubscript{B} receptor deficiency itself contributes to the abolition of the sex differences in ET\textsubscript{B}-deficient rats.

In conclusion, ET\textsubscript{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\textsubscript{B} receptor- and estrogen receptor-signaling systems remains unclear. In addition, the lack of the vasoprotective effects of estrogen and the augmentation of ET\textsubscript{A} receptor-mediated action seem to be responsible for the abolition of sex differences in ET\textsubscript{B} receptor deficiency.

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**Figure legends**

Figure 1

Light micrographs of the injured carotid artery at 2 weeks after balloon injury in the wild-type (A-D) and ET<sub>B</sub>-deficient (E-H) rats. A and E: males, B and F: intact females, C and G: OVX, and D and H: OVX + E₂ rats (Elastica Van Gieson staining, magnification, x100).

Figure 2

Neointima/media ratio of the injured arteries in wild-type and ET<sub>B</sub>-deficient rats at 2 weeks after balloon injury. Data are expressed as the mean ± S.E.M. (n=5-6). ** P < 0.01, compared with intact female group of wild-type rats. †† P < 0.01, compared with OVX group of wild-type rats. # P < 0.05, ## P < 0.01, compared with the corresponding wild-type rats.

Figure 3

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

Figure 4

Neointima/media ratio of the injured arteries in wild-type and ET<sub>B</sub>-deficient rats at 2 weeks after balloon injury and the effect of J-104132. Data are expressed as the mean ± S.E.M. (n=5-6). ** P < 0.01, compared with control group of the same genotype and sex.

Figure 5
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.
Table. Comparative Data on Body Weight, Uteri Wet Weight, Systolic Blood Pressure, and Neointima and Media Area

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<th>SBP (mmHg)</th>
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<td>116 ± 4</td>
<td>0.063 ± 0.009</td>
<td>0.103 ± 0.002</td>
</tr>
<tr>
<td>OVX</td>
<td>283 ± 7†</td>
<td>0.152 ± 0.013**</td>
<td>120 ± 4</td>
<td>0.120 ± 0.016*</td>
<td>0.105 ± 0.003</td>
</tr>
<tr>
<td>OVX + E₂</td>
<td>258 ± 3</td>
<td>0.336 ± 0.004††</td>
<td>117 ± 2</td>
<td>0.073 ± 0.004</td>
<td>0.106 ± 0.003</td>
</tr>
<tr>
<td><strong>ETB-deficient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>369 ± 8**</td>
<td>--</td>
<td>141 ± 3†</td>
<td>0.214 ± 0.016* #</td>
<td>0.137 ± 0.004**</td>
</tr>
<tr>
<td>female</td>
<td>246 ± 4</td>
<td>0.438 ± 0.024</td>
<td>140 ± 2##</td>
<td>0.158 ± 0.006##</td>
<td>0.103 ± 0.004</td>
</tr>
<tr>
<td>OVX</td>
<td>279 ± 4*</td>
<td>0.141 ± 0.015**</td>
<td>138 ± 1##</td>
<td>0.178 ± 0.017##</td>
<td>0.130 ± 0.009##</td>
</tr>
<tr>
<td>OVX + E₂</td>
<td>254 ± 4†</td>
<td>0.372 ± 0.019††</td>
<td>138 ± 2##</td>
<td>0.177 ± 0.013##</td>
<td>0.123 ± 0.005*</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM (n = 5 or 6). SBP, systolic blood pressure; OVX, ovariectomized; E₂, 17β-estradiol.

*P < 0.05, **P < 0.01 compared with the females of the same genotype group; †P < 0.05, ††P < 0.01 compared with the OVX rats of the same genotype group; #P < 0.05, ##P < 0.01 compared with corresponding wild-type rats.
Figure 1

(A) male wild-type

(B) female wild-type

(C) OVX wild-type

(D) OVX + E2 wild-type

(E) male ETβ-deficient

(F) female ETβ-deficient

(G) OVX ETβ-deficient

(H) OVX + E2 ETβ-deficient
Figure 2

Neointima/Media ratio

***

††

# ####

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Figure 3

Plasma ET-1 Level (pg/ml)

- Male wild-type
- Female wild-type
- Male ET<sub>B</sub>-deficient
- Female ET<sub>B</sub>-deficient

* * *
Figure 4

Neointima/Media ratio

wild-type ET\textsubscript{B}-deficient male

wild-type ET\textsubscript{B}-deficient female

control J-104132

**

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Figure 5

Neointima/Media ratio

- ** vehicle (n=5)
- A-192621 (n=4)
- ABT-627 (n=4)
- J-104132 (n=5)

male    female

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